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BACTERIOLOGY

In relation to Clinical Medicine

THEORETICAL AND APPLIED

For Students, Laboratory Workers and Practitioners in Medicine and Public Health

BY

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With 276 Illustrations (including 42 coloured)

⁂

PRINTED AND PUBLISHED AT
THE "STATESMAN" PRESS
CALCUTTA

1935

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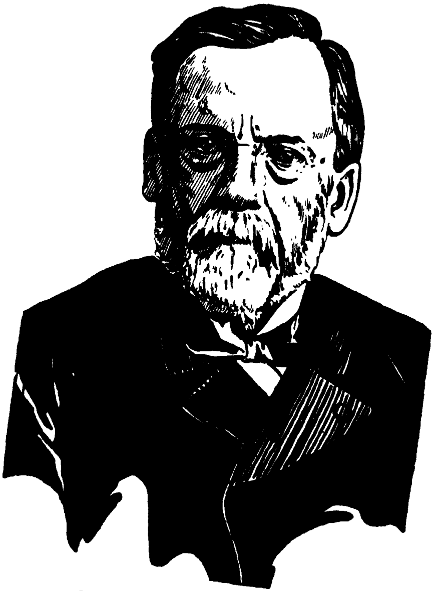
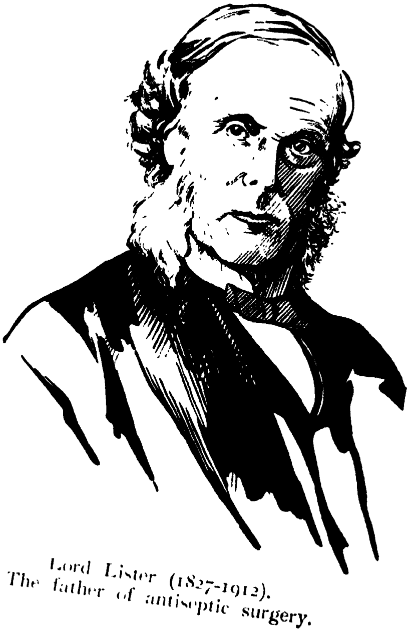
Dedicated to the memories of

Anthony van Leeuwenhoek (1632-172
Inventor of the microscope.



Louis Pasteur (1822-95).
The father of microbiology and preventive medicine.

Robert Koch (1843-1910).
The father of modern bacteriology.



Lord Lister (1827-1912).
The father of antiseptic surgery.

PREFACE

This book is intended to correlate the modern ideas of bacteriology and immunology with the study and diagnosis of human diseases and also to some extent their rational modes of treatment. In the following pages, endeavour has been made to present in a concise, intelligible and readable manner such facts which every student should know before he passes from the pre-clinical or early clinical period to the study of the various practical branches of medicine, surgery and obstetrics. The subjects of bacteriology and immunology have made such rapid strides during the last few years and their literature has assumed such an enormous volume that it is impossible for a general student to keep pace with this progress. It is for this reason that we have been careful to omit all unnecessary details and to present to the already over-burdened students only such facts which are absolutely essential for their study.

Although this work is primarily written for advanced students preparing for the degree of Bachelor of Medicine and Diplomas of Public Health and Tropical Medicine, we hope that it will be no less useful to the present generation of practitioners and laboratory workers. Unlike other text books of bacteriology, much more space has been allowed to discuss in detail the various pathological processes induced by bacterial infections and the methods of their diagnosis, as it is our firm belief that without such discussions the study of this important branch of science loses much of its interest and becomes a matter of theoretical importance.

Modern nomenclature has been used throughout the text. But in case such a wholesale innovation may cause any confusion in persons not accustomed to the more current terminology, the latter has also been incorporated for ready reference.

It is needless to emphasise the value of good illustrations in a scientific book of this nature and we have spared nothing to fulfil this end. Every one of them has been specially chosen to assist the reader to comprehend what he reads in the text. Most of them have been taken from actual specimens and preparations which have been studied in the laboratory and wherever possible, coloured diagrams have been introduced to impress the reader. In this connection, we beg to express our grateful thanks to the publishers of a number of well-known books and periodicals for their courtesy in granting permission to use some illustrations from their works. All these have been fully acknowledged in the text.

This book will be found to be a new departure as we have not included some sections on Parasitology and Mycology in the text. This has been done on purpose as we are definitely of opinion that such an admixture is not a very happy one because it only tends to foster a haphazard study of these subjects which in the tropics should be taken up separately.

All recent works on the subject have been freely consulted in order to maintain a uniform and up-to-date standard. In the general arrangement of the text, we have followed the line adopted by the Medical Research Council of England. Every emphasis has, however, been laid on many important features which are of special interest to the students and practitioners of the tropics.

We are particularly indebted to Dr. D. M. Chatterjee for his assistance in reading and correcting the proof sheets and also to the members of the staff of the Department of Pathology, Medical College, Calcutta, for their ungrudging help, suggestions and co-operation.

Indebtedness is also expressed to Capt. C. L. Pasricha, I.M.S., Professor of Bacteriology, Calcutta School of Tropical Medicine and Hygiene, for his assistance in preparing the illustrations on Plague, to Dr. B. P. Tribedi and Dr. S. C. Chandra for their assistance in preparing the sections on serological methods, to Dr. J. L. Dawn for his assistance in preparing the section on water bacteriology, to Mr. P. Chatterjee, F.R.C.S., for lending illustrations on carbuncle and facial cellulitis and to Dr. K. B. Ghosh for the skiagram of apical tooth abscess.

We are indebted to Mr. Susil Bhattacharya for the original drawings and photographs and to Mr. L. E. Lane of the Statesman, Ltd., for his masterly way of preparing the blocks for illustrations in the text.

We also express our deep appreciation of the highly efficient, painstaking and prompt assistance rendered by Mr. M. S. Scurrah of the Statesman, Ltd., without whose valuable suggestions and co-operation, it would not have been possible to publish the book in its present form.

M. N. DE.
K. D. CHATTERJEE.

CALCUTTA.
AUGUST, 1935.

FOREWORD

Dr. M. N. De and Dr. K. D. Chatterjee have asked me to write a short foreword to this admirable volume. I have had the pleasure of knowing Dr. De and his work for the past fifteen years ; he is a most conscientious and capable worker, and an admirable teacher and professor, popular with his students, and most painstaking in his work.

The book is admirably written. It is sufficient and contains a wealth of information, yet is not overloaded. A special feature of the work is that almost as much pathology as bacteriology is incorporated in it. The illustrations are of special value, and above all, the splendid colour plates, most of which are original. Much recent work in both bacteriology and pathology has been included, which is omitted from older and smaller textbooks. Finally, the style of publication is far and above the usual standard of Indian publications and the *Statesman* is to be warmly congratulated on this.

One agrees with the authors that it is better in such a work not to include the subject of parasitology ; it is now so vast that it is better dealt with in separate publications. Throughout the book the teaching pays special attention to tropical conditions and tropical diseases.

The authors are to be congratulated on the production of a work which will appeal to the medical student, the post-graduate student and the laboratory worker alike. One wishes the book the success that it so richly deserves.

R. KNOWLES,
C.I.E., Lt.-Colonel, I.M.S.

Calcutta School of Tropical Medicine.
12th August, 1935.

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MEDICAL BACTERIOLOGY

CHAPTER I

INTRODUCTION

Bacteria are minute unicellular vegetable organisms devoid of chlorophyll. They multiply by simple transverse division or fission; hence called Schizomycetes or fission fungi (Gr. *Schizein,* to cleave; ***myketes,*** mushrooms or fungi). The systematic position of bacteria is shown in the following table.

Plant kingdom

Cryptogams (flowerless plants)

Thallophyta (no distinction between stem, root and leaves).

Algæ (with chlorophyll)	Lichens (symbiosis between algæ and fungi)	Fungi (without chlorophyll)

Schizomycetes (reproduction by fission)	Blastomycetes (reproduction by budding) e.g. Yeasts	Hyphomycetes (reproduction by spores) e.g. Moulds (Oidia)

The study of Hyphomycetes and Blastomycetes comprises the science of *MYCOLOGY* whereas the study of Schizomycetes comprises the science of *BACTERIOLOGY.*

CLASSIFICATION OF BACTERIA OR SCHIZOMYCETES

For practical purposes the bacteria may be divided in the following way:—

Bacteria (Schizomycetes)

Lower bacteria	Higher bacteria
(1) Cocci	Actinomyces (Streptothrix)
(2) Bacilli	*e.g.* Actinomyces maduræ
(3) Spirillæ	Actinomyces bovis
(a) Vibrios	
(b) Spirillæ	
(c) Spirochætes	

Filter-passing viruses:—Apart from the above group of living organisms there still remains a considerable number, which owing to their minute size are either beyond the limits of visibility or barely visible with the highest magnifications of the microscope. Hence such organisms are often termed ultra-microscopic viruses. These organisms, due to their small size or plasticity, are able to pass through the pores of a porcelain filter and when such a filtrate is injected into a susceptible animal, it will produce disease, proving thereby that the infecting agent is a filter-passing virus.

MORPHOLOGICAL STUDY OF SCHIZOMYCETES (BACTERIA)

SHAPE:—The bacteria differ in their shape from one another and can be divided into 3 main groups.

(1) ***Cocci:***—These are dot-shaped organisms and are more or less spheroidal or rounded in shape, their longitudinal and transverse diameters are almost equal. (Fig. 1a).

(2) ***Bacilli:***—These are often called rod-shaped organisms but truly speaking, they are cylindrical and the longitudinal axis is at least twice their transverse diameter (Fig. 1b).

(3) ***Spirillæ:***—The organisms are bent and twisted either forming (a) a single curve when it is called a vibrio (Fig. 1c) or (b) a series of curves when it is called a spirillum (Fig. 1d) or spirochæte (Fig. 1e), the difference between the two, however, depending on the rigidity or flexibility of the curves.

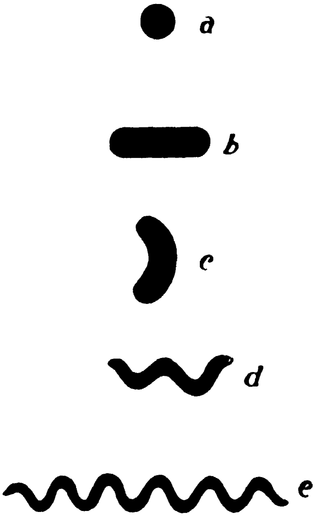


Fig. 1.
Various shapes of bacteria.
a. spherical (coccus); b. cylindrical (bacillus); c. curved like a comma (vibrio); d-e, a series of curves (d. spirillum; e. spirochæte).

SIZE:—Bacteria are all very minute organisms; they vary much in size and the average diameter of cocci may be taken as 1 micron. A micron (μ) is $\frac{1}{1000}$ of a millimetre or $\frac{1}{25000}$

of an inch. The average measurement of bacilli may be taken as 2 to 4 μ in length and about 1 μ in breadth. Of the common pathogenic bacilli, the bacillus of influenza is a very small one and measures about 0.75 μ in length by 0.2 μ in thickness. The largest bacillus recorded is B. butschlii of Schaudin (50 to 80 μ long and 4 to 5 μ wide). The spore-bearing bacilli are, as a rule, somewhat larger in size. The spirillæ are usually much longer.

ARRANGEMENT: —This mainly depends on the method of division.

Cocci:—According to the nature of division in successive planes the following arrangements are possible:—

(1) Division taking place in one plane only:—
(a) It may form a chain e.g. Streptococcus (Fig. 2a) or (b) it may be grouped in pairs (Diplococcus) e.g. Diplococcus pneumoniæ (Pneumococcus) Fig. 2b, Neisseria gonorrhœæ (Gonococcus) Fig. 2c, Neisseria meningitidis (Meningococcus) Fig. 2d, Neisseria catarrhalis (M. catarrhalis) Fig. 2e, etc.

(2) Division taking place in two planes at right angles to each other (Tetracoccus) e.g. Gaffkya tetragena (M. tetragenus) (Fig. 2f).

(3) Division taking place in three planes forming cubes e.g. Sarcina ventriculi (Fig. 2g).

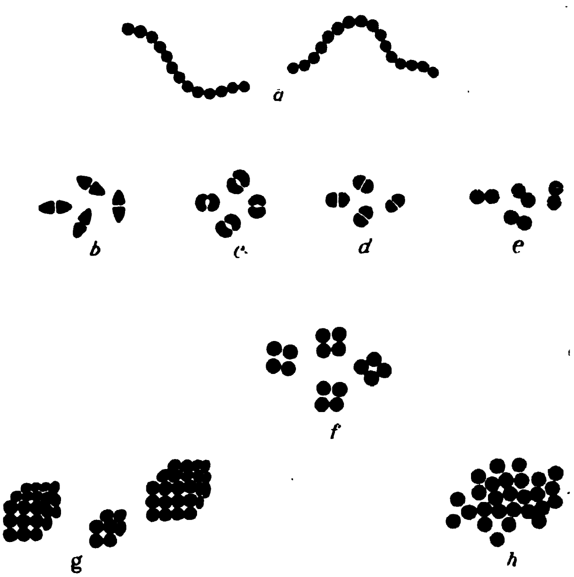


Fig. 2.
Arrangement of cocci.
a, cocci in chains (Streptococcus); b-e, cocci in pairs (b, D. pneumoniæ; c, N. gonorrhœæ; d. N. meningitidis; e, N. catarrhalis); f, cocci in tetrads (G. tetragena); g, cocci in cubes (Sarcina); h, cocci in groups (Staphylococcus).

(4) Division occurring in any plane forming groups or bunches e.g. Staphylococcus (Fig. 2h).

Bacilli:—Most of the bacilli are arranged singly (Fig. 3a). They may, however, (a) form chains (Streptobacillus) e.g. B. anthracis (Fig. 3b), B. subtilis or (b) grouped in pairs (Diplobacillus) e.g. Klebsiella pneumoniæ (Friedländer's pneumobacillus) Fig. 3c, Hæmophilus conjunctivitidis (Koch-Weeks bacillus), Hæmophilus lacunatus (Morax-Axenfeld bacillus).

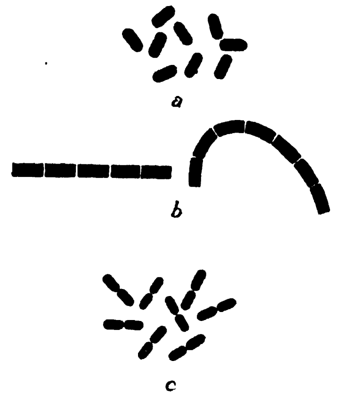


Fig. 3.
Arrangement of bacilli.
a, bacilli arranged singly (E. dysenteriæ); b, bacilli in chains (B. anthracis); c, bacilli in pairs (K. pneumoniæ).

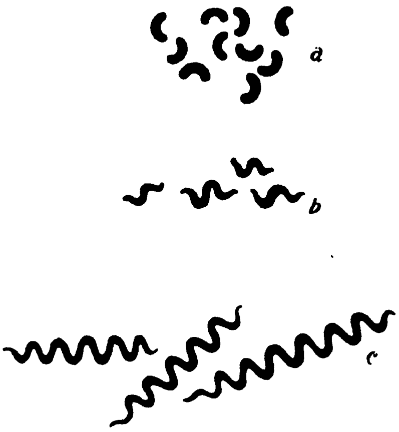


Fig. 4.
Arrangement of spirillæ.
a, vibrios; b, spirillæ; c, spirochætes.

Spirillæ:—These are arranged singly (Fig. 4a, b & c).

INTERNAL STRUCTURE:—This may be studied in two ways.

(i) ***In unstained condition,*** bacteria appear as transparent, colourless, refractile and apparently homogeneous bodies.

(ii) ***In fixed and stained condition,*** the internal structure can be more definitely studied, as the different constituents of the bacterial body have varying affinities for different dyes employed in the staining; it thus serves as a sort of micro-chemical test. Moreover, different kinds of bacteria may show differences in their general staining reaction which may be applied practically as a diagnostic help and as a useful guide to their classification.

Bacteria consist mainly of a mass of protoplasm, surrounded by a delicate cell-membrane. The internal structure comprises, (a) nuclear apparatus and (b) intra-cellular granules.

(a) **Nuclear apparatus:**—Regarding the presence of a nucleus in bacterial cells, the following different views are held:—

i. That the entire bacterial body consists of nuclear material (*Zettnow*).

ii. That there is a definite nucleus and the nuclear material is concentrated inside the endoplasm.

iii. That inside the endoplasm, there is a reticular network, between the meshes of which lie granules of chromophilic nature (*Butschli*).

Thus, according to this view, the nuclear granules are scattered throughout the cytoplasmic network (Fig. 5).

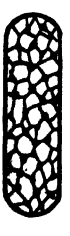


Fig. 5.
Disposition of the nuclear material inside the bacillary body.

Note:—The consensus of opinion about the presence of nuclear material is, that it is present in some form or other inside the bacterial body.

(b) **Intra-cellular granules:**—In some instances, inside the bacterial body e.g. in Corynebacterium diphtheriæ, there are present granules which have a strong affinity for nuclear stains. These are called *volutin* or *metachromatic granules.* They are also known as *Babes-Ernst granules,* after the name of their discoverers. These form no essential part of the living constituents of the cell; they are only "metaplasmatic granules, particles of some substance concerned in cell metabolism." They contain a high proportion of nucleoprotein. Their number and size vary according to the medium in which the organisms grow. They take no part in cell division or sporulation.

SPORES (ENDOSPORES):—This was first studied in detail by Koch (1876) in the case of Bacillus anthracis. At the very outset, a distinction must be made between endospores and arthrospores. *Arthrospores* are not true spores but are only degenerated and involution forms of the bacteria developing in cultures. They are notably seen in Corynebacterium diphtheriæ. They are also never more resistant than the ordinary vegetative cells. *Endospores* are true spores and are always intracellular. These are very resistant to heat, desiccation, chemical and other deleterious agents.

Mode of formation:—Three stages can be observed in the development of spores (Fig. 6a, b, c, d, e, f).

First stage:—Localised accumulation of chromatin-like material which stains deeply with nuclear stains (Fig. 6a, 6b).

Second stage:—Formation of an impervious wall or membrane round the spore body. At this stage stain can not penetrate unless specially treated (Fig. 6c, 6d).

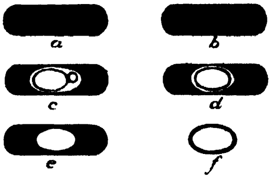


Fig. 6.
Mode of formation of endospores, examined in an unstained condition.

Third stage:—Maturation of spores occurs when they appear, in an unstained specimen, as highly refractile ovoid bodies (Fig. 6e). After it is fully developed, the body of the bacillus disappears and the spore is left as a free structure (Fig. 6f).

It should be remembered that the spore-forming bacteria belong to the Bacilli group and are monosporous each forming one spore only. Spores are never developed while bacilli are inside the animal body, except in the case of anaerobic bacteria (Clostridia).

Varieties:—The position of the spore in the bacterial body is usually constant for one and the same species. Hence it serves as an important distinguishing point which can be utlised for the differentiation of certain bacterial species. Thus, according to their situation within the bacterial bodies, spores may be termed—(a) *equatorial* (Fig. 7a), when they are situated in the central part of the bacterial body e.g. in B. anthracis, (b) *terminal* (Fig. 7b), when they are situated at one end of the bacillary body e.g. in Clostridium tetani and Clostridium botulinum or (c) *subterminal* (Fig. 7c), when they are situated between the centre and the end of the bacillus e.g. in Clostridium sporogenes.

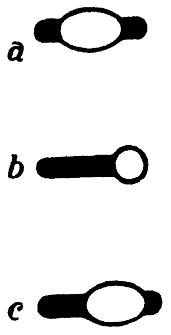


Fig. 7.
Situation of endospores.
a, equatorial or central spore (B. anthracis); b, terminal spore (Cl. tetani); c, subterminal spore.

The shape of the spore may be spherical, oval or ellipsoidal. The diameter of a mature spore is often larger than that of the bacillary body which contains it. Hence the cell is distorted and according to the shape of spore and its situation in the bacterial body, various forms are presented which are often characteristic of a particular organism. Thus the bacteria may show the appearance of

Drum-stick—when the spores are spherical and terminal e.g. Cl. tetani;

Tennis-racket—when the spores are oval and terminal e.g. Cl. tertium;

Club-shaped—when the spores are oval and subterminal e.g. Cl. sporogenes;

Barrel or spindle-shaped—when the spores are equatorial and oval or ellipsoidal e.g. Cl. bifermentans and B. anthracis.

Purpose of spore formation:—It is simply a resting stage of the bacterial cell in which it is far more resistant to adverse environmental conditions than it is in the ordinary vegetative form. The process is analogous to encystment and it is a protective mechanism for the safety of the species against the injurious effects of heat, light, drying, chemicals, want of food and oxygen and change in the reaction of the medium in which it grows. Its resistance is enormous as is shown in the following table:—

RESISTANCE OF B. ANTHRACIS

	Vegetative Forms	Spores
10 per cent. phenol	Killed in 20 minutes.	Resistant.
50 per cent. phenol	Killed at once.	Killed in 20 minutes.
Steam and boiling	Killed at once.	Killed in 10 to 12 minutes.

The great resistance of spores is probably due to the following:—

(i) Presence of a thick and very resistant cell-membrane,

(ii) Low water content—This enables them to resist a high temperature, as the lower the water content of a solution of albumin, the greater is the temperature necessary to coagulate it.

Germination of spores:—When placed under favourable conditions of temperature, moisture and nutrition, spores develop into vegetative forms. This may take place with or without rupture and shedding of the spore-envelope. The commonest method of germination is with rupture and shedding. The process consists of an elongation of the spore-body with loss of the highly refractile character and resisting power. Then, there occurs a rupture of the wall and the bacillus slips out, leaving the empty spore capsule. Such rupture of the wall and slipping out of the bacillus may take place in one of the two ways. Thus when it occurs at the centre, it is called *equatorial germination* (Fig. 8a) and when it occurs at one of its poles, it is called *polar germination* (Fig. 8b).

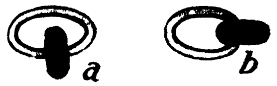


Fig. 8.
Germination of spores.
a, equatorial germination; b, polar germination.

Staining reactions:—Special stains are necessary for demonstration

Practical application of the knowledge of spore formation:—The knowledge of the various properties of the spores described above is very important in practical life. We, as medical men, have frequently to deal with infective lesions caused by such spore-bearing organisms as Cl. tetani, B. anthracis and organisms of gas-gangrene. During such occasions, we should be very careful to handle them and to take all possible care to dispose of all the infected materials properly not only for our own safety, but also to prevent the spread of the contagion to others. All articles, such as instruments, dressings, beddings, wearing apparels, etc. should never be used again unless fully sterilized by high pressure steam sterilisers. If possible, they should all be scraped off and burnt. In a crowded hospital, the danger of injudicious disposal of materials suspected to contain spores is very great indeed and it is for this reason that these cases should always be segregated and all articles used for them be kept separate. In dressing them, rubber gloves should be used and the latter should be thoroughly sterilised by prolonged boiling and subsequently kept immersed in a solution (1 in 1000) of bichloride of mercury. After finishing the case, they should be destroyed.

When a cattle is suspected to have died of anthrax infection, it should either be incinerated or buried underground with sufficient amount of quick-lime, covering it all round.

CAPSULES:—Under certain conditions, many bacteria may develop a definite outer envelope to the bacterial body which is called a *capsule*. By the ordinary methods of staining, the capsule usually appears as a clear unstained zone, surrounding the bacterial cell. The capsules can however be seen when stained, after suitable fixing and mordanting. It is said to be formed as a result of an alteration in the outer layer of the cell-wall, which produces a marked thickening and change in consistency. Thus, it appears to be surrounded by a mucilaginous envelope appearing as a halo round the bacteria (Fig. 9).

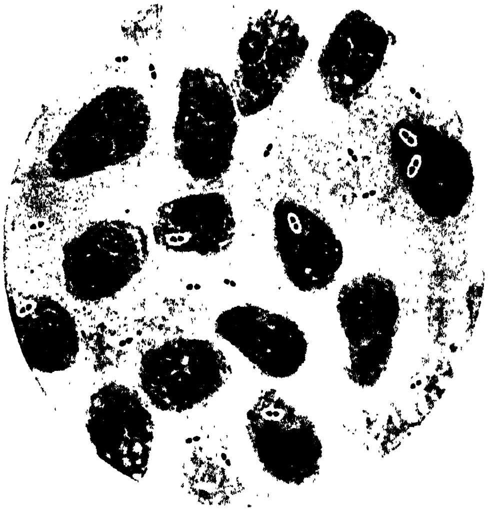


Fig. 9.
Capsules of D. pneumoniæ appearing as an unstained halo, in a smear preparation of pus from a case of empyema thoracis

Nature:—*Meyer* and *Zettnow* have pointed out that the capsular substance is an active secretion. They have also demonstrated that when bacteria are grown in a medium containing a particular carbohydrate, marked capsule-formation will take place. Hence it appears that a capsule does not form an essential part in the architecture of the bacterial cell, but it may be regarded as a result of the vital response to environmental requirements. Capsulated or non-capsulated means the readiness or frequency with which this reaction to environment takes place.

Capacity for capsule formation: All bacteria may form capsules in variable degrees but they are unequally developed in different species, and under different circumstances. The bacteria which exhibit marked capsule formation are Diplococcus pneumoniæ (Pneumococcus), Klebsiella pneumoniæ (Friedländer's pneumobacillus) and Clostridium welchii (B. ærogenes capsulatus). Capsules are usually formed inside the animal body. They are not developed in artificial cultures unless the media are enriched with some body fluid.

Purpose of capsule formation:—The presence of a capsule appears to be associated with increased virulence of the organism as the former protects it from the destructive effects of the blood and tissues of the host.

Hence this capsular equipment enables the germ to multiply rapidly in the tissues of the animal host producing serious effects.

Staining reactions:—Special stains are necessary for the demonstration of capsules (Page 26.)

FLAGELLA:—These are long contractile hair-like processes arranged in various ways round the bacterial body. They are very slender but much longer than the bacterial cells. The organisms possessing flagella belong mostly to the bacilli and spirillæ group. Cocci and spirochætes do not exhibit any flagella.

Nature:—They are not demonstrable by ordinary methods of staining but require special methods for their study. When rendered visible by such methods, they are found to be delicate elongations of the protoplasm of the bacterial bodies. The central portion of the flagellar thread appears to pass through the cell membrane and is in direct communication with the cytoplasm, and possibly with a granule of chromatin (Fig. 10); this being comparable to the blepharoplast of the flagellated parasites.

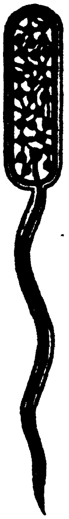


Fig. 10.
Relation of flagellum to bacillary body.

Disposition and arrangement:—Bacteria may be classified according to the arrangement of their flagella in the following ways:—

1. *Atrichate* possessing no flagella e.g. Eberthella dysenteriæ (B. dysenteriæ).

2. *Monotrichate* —possessing one flagellum at one pole only, a single terminal flagellum (Fig. 11a) e.g. Vibrio comma (V. choleræ).

3. *Amphitrichate*—possessing one flagellum at each pole (Fig. 11b).

4. *Lophotrichate* —possessing a bunch of flagella at one or both poles (Fig. 11c) e.g. Spirillum minus of rat-bite fever.

5. *Peritrichate*— the flagella are arranged indiscriminately all round the bacterial cell, multiple peripheral flagella (Fig. 11d) e.g. in Eberthella typhi (B. typhosus), Escherichia coli (B. coli), Clostridium tetani (B. tetanus), Clostridium botulinum (B. botulinus), etc.

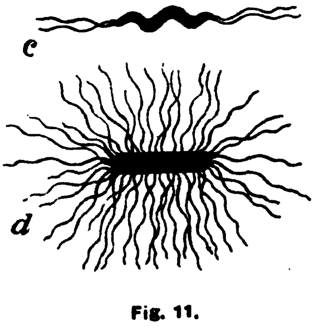
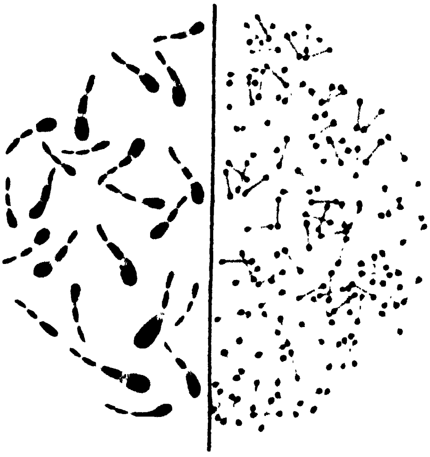


Fig. 11.
Disposition and arrangement of flagella.
a, monotrichate (V. comma); b, amphitrichate; c, lophotrichate (Sp. minus); d, peritrichate (E. typhi).

Note:—It should be remembered that only one type of arrangement and disposition of the flagella is possible for any one type of organism and it always remains constant for that particular species. For example, all the strains of Eberthella typhi exhibit only the peritrichate arrangement of their flagella and no other variation from this is possible.

Chemical composition:—Flagella differ from bacterial cells in chemical composition. They produce a special group of agglutinin which is thermolabile as opposed to somatic or body agglutinin which is thermostable. This will be described in detail under the chapter on agglutinins.

Purpose:—The flagella give to the organisms their power of locomotion in a fluid medium. Bacterial motility which is due to the presence of these flagella, can be studied by moist cover-glass or hanging-drop preparations. In this connection, one should note the difference between true motility (the organism changing its position in the field), Brownian movement (a to-and-fro oscillation of the organism) and movement due to the current of a fluid (all bacteria are seen to move in one direction).



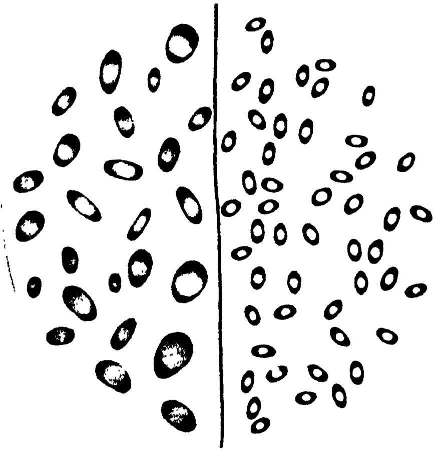
Normal forms

Fig. 12.

Corynebacterium diphtheriae.

Staining reactions:—Special stains are necessary for the demonstration of flagella (Page 30).

INVOLUTION FORMS:—These are frequently found in old cultures. Some of them are very typical of the bacterial species in which they occur. These represent the degenerative and distorted forms of the organisms and they appear as the cultures grow old and the environmental conditions become unfavourable. The typical examples are, Corynebacterium diphtheriae (B. diphtheriae), Pasteurella pestis (B. pestis; Bacillus of plague) and Neisseria meningitidis (Meningococcus) (Figs. 12, 13 and 14). Sometimes in Eberthella typhi ng filaments resembling fungus may appear. These filamentous forms are not involution forms but are due to del in cell division and sub quent separation.



Involution forms. Normal forms.

Fig. 13.

Pasteurella pestis.

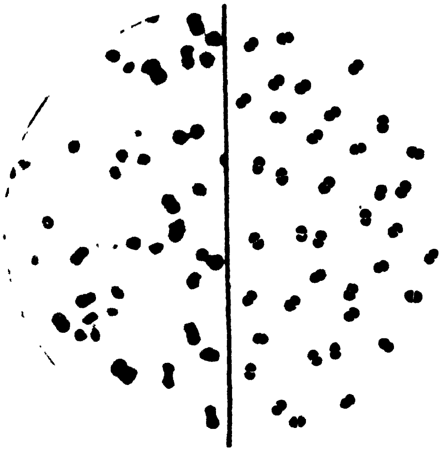


Fig. 14.
Involution forms. Normal forms.
Neisseria meningitidis.

REPRODUCTION:—Bacteria multiply usually by simple binary fission and such division is known as asexual multiplication. In the presence of a liberal food supply and other conditions for growth being satisfactory, the organisms increase in size and become mature. A constriction appears in the middle of the bacterial body and ultimately each cell divides into two daughter cells. The new individuals may separate almost immediately or may remain in contact for a longer or shorter period, depending on the character of their cell envelopes. In coccal forms, division occurs in any diameter, but in bacillary and spiral forms, the division always occur in the transverse diameter, i.e. at right angles to their long axies. The bacteria multiply with such an enormous rapidity that a single individual may, in 24 hours, give rise to more than 15 millions of organisms.

Formation of Cell-groups:—After division, the daughter cells may at once separate or remain attached to one another for a variable period forming little groups. The forms of these cell-aggregates will depend on the planes in which successive divisions take place and also the number of cell divisions taking place before separation. Such groupings are very characteristic of particular species of bateria and afford a valuable guide in their identification. As in Cocci the division takes place in any plane, whereas in Bacilli and Spirillae the division takes place only in the transverse diameter, the former show larger variety of groupings than the latter.

Types of Cell-aggregates:—

(a) Coccal forms:

(1) When division occurs in such a way that the daughter cells remain attached to each other for a short period, the bacterium in question may be termed a Diplococcus (Fig. 15a).

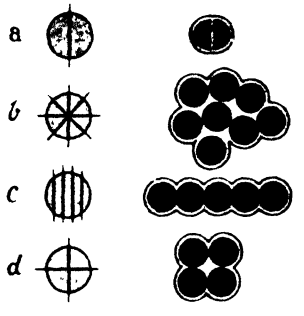


Fig. 15.
Planes of division and resulting cell-aggregates.

a & g, in one plane (Diplococcus and Diplobacillus); d, in two planes at right angles to each other (Tetracoccus); e, in three planes at right angles to one another (Sarcina); b, in successive planes irregularly (Staphylococcus); c & f, in successive planes parallel to one another (Streptococcus and Streptobacillus); h, C. diptheriæ (L or V forms).

(2) When an organism divides rapidly and successively in any plane without immediate separation of the daughter cells, it forms an irregular colony which may be compared to a bunch of grapes. Such an organism is called Staphylococcus (Fig. 15b & 16a).

(3) When the bacterium divides in planes parallel to one another and the daughter cells do not separate after division, it produces the appearance of a chain of beads and is then referred to as Streptococcus (Figs. 15c & 16b).

(4) In some cases, the cells remain attached while two divisions occur, the second at right angles to the first, forming a group of 4 cocci and this is described as Tetracoccus. (Figs. 15d & 16c).

(5) When the organism divides in three planes, each of which is at right angles to the other two, the daughter cells remaining united during successive divisions, it produces a typical cell-aggregate consisting of 8 cocci which may be compared to cubical blocks or packets. Such an organism may be designated as a Sarcina (Figs. 15e & 16d).

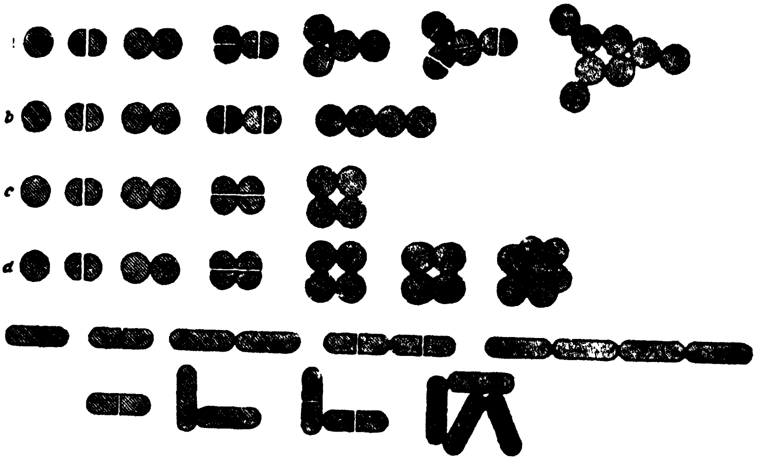


Fig. 16.

Stages in the cell division.

a, Staphylococcus; b, Streptococcus; c, Tetracoccus, d, Sarcina; e, B. anthracis; f, C. diphtheriæ (L or V forms and Chinese letter arrangement).

(b) **Bacillary forms:**—The only possibility in these cases is the adherence of two or more cells in pairs (Diplobacillus) or in chains (Streptobacillus). The following are the typical examples:—

Of Streptobacillus (Figs. 15f & 16e)—B. anthracis (long chains) and B. subtilis (short chains).

Of Diplobacillus (Fig. 15g)—Klebsiella pneumoniæ (Friedländer's pneumobacillus), Hæmophilus conjunctivitidis (Koch-Weeks bacillus) and Hæmophilus lacunatus (Morax-Axenfeld bacillus).

In Corynebacterium diphtheriæ, the daughter cells after division remain attached to one side of the body (Figs. 15h & 16f). It acts as a hinge on which the daughter cells swing and come to lie in varying angles producing L or V forms and further division occurs when they are in the above position. This asymmetrical division gives rise to "Chinese-letter" arrangement.

(c) **Spiral forms:**—No characteristic cell-groupings are seen with this type of bacteria. Sometimes the two daughter cells may be found to remain attached end to end. This may explain the appearance of S-forms in V. comma.

CHAPTER II

STAINING REACTIONS

Bacteria are composed of a highly refractile protoplasm, and unless special methods of illumination, such as dark-ground illumination, is employed, they cannot be seen in an unstained condition. It is for this reason that stains are necessary to demonstrate the morphological structure of a bacterium. The most commonly employed bacterial stains are basic aniline dyes. Some bacteria take this stain readily, while others require the use of a mordant, weak alkalis or prolonged heating which increases the penetrating power of the stain. They also differ in their staining affinities, which enables us to differentiate one organism from others. Thus staining is of importance not only for the recognition of bacteria but also for their classification. The following stains are commonly used.

1. ***Ordinary aniline stains:***—These are single colour stains and are employed for the quick diagnosis of the morphology of bacteria.

2. ***Differential stains:***—These are more of the nature of micro-chemical tests than methods of demonstrating structure. The two common examples are:—(a) Gram's stain and (b) Acid-fast stain.

3. ***Special stains:***—These are used for the demonstration of capsules, flagella and endospores of bacteria and of metachromatic granules of Corynebacterium diphtheriæ.

4. ***Compound stains:***—These are mixed dyes or compounds formed by the interaction of two or more dyes. The most commonly employed stain under this group is one originally introduced by Romanowsky. The various modifications of Romanowsky's stain are (*a*) *Leishman's Stain,* (*b*) *Wright's Stain,* (*c*) *Giemsa's Stain* and (*d*) *Jenner's Stain.*

5. ***Vital stain:***—This is a method by means of which a dye substance is applied to cells or tissues while still in the living state. Two types of vital staining are known:—(*a*) *Intra-vitam,* when the dye-stuff is injected into the living animal and (*b*) *Supra-vitam,* when portions of living tissues are brought in contact with the dyes outside the body.

ORDINARY ANILINE STAINS.

Most bacteria take this stain. These stains are derived from aniline which is a coal-tar product and they comprise the following:—

(i) ***Basic aniline dyes:***—These have affinity for nuclear material and are suitable for staining bacteria. They include the following:—

Violet stains—Crystal violet, gentian violet, methyl violet.
Blue stains—Methylene blue, toluidin blue, thionin.
Red stains—Basic fuchsin, saffranin.
Brown stains—Bismarck brown, chrysoidin.

(ii) ***Acid aniline dyes:***—These stain the cytoplasm and are not suitable for bacteria. They are used in bacteriology for the purpose of counter-staining and include *eosin, acid fuchsin, picric acid, brilliant green, congo red, etc.*

Different bacteria stain in different ways and certain bacteria exhibit characteristic reactions which enable them to be identified. Thus, the bacteria may behave in one of the following ways:—

(1) **Uniform staining** (Fig. 17)—The whole bacterial body is uniformly coloured, e.g., organisms of the coli-typhoid group.

(2) **Polar staining** (Fig. 18)—Only the ends of the bacilli are coloured, the central portions remaining almost colourless, e.g., Pasteurella pestis (B. pestis) and Hæmophilus influenzæ (B. influenza).

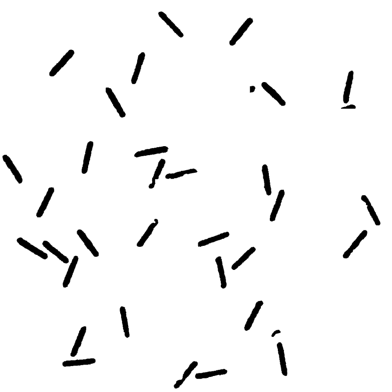


Fig. 17.
L. dysenteriæ showing uniform staining.

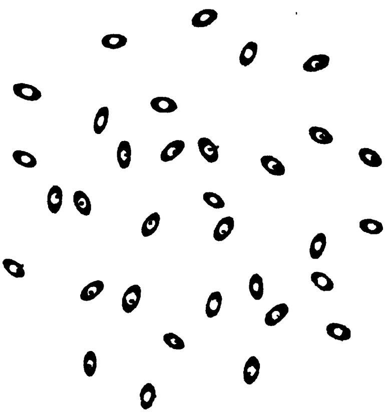


Fig. 18.
P. pestis showing polar staining.

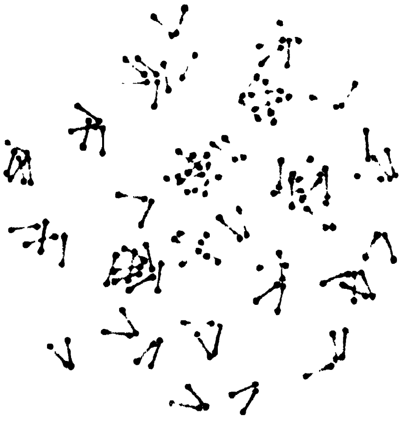


Fig. 19.
C. diphtheriæ showing metachromatic staining.

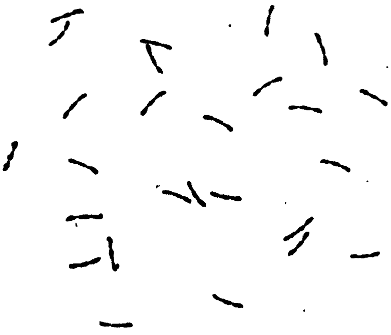


Fig. 20.
M. tuberculosis showing beaded or granular staining.

(3) **Metachromatic stain** (Fig. 19)—The bacilli stain irregularly owing to the presence of certain granules which have much more affinity for the stain, e.g., Corynebacterium diphtheriæ.

(4) **Granular stain showing beaded appearance** (Fig. 20)—The bacilli stain unevenly throughout their lengths, so that beaded forms occur. The cause of such irregular staining is not due to the presence of metachromatic granules which are absent in this case. It is due to unequal distribution, throughout the cells, of those constituents of the cytoplasm which take up the stain, e.g., Mycobacterium tuberculosis (B. tuberculosis).

DIFFERENTIAL STAINS

I. GRAM'S STAIN

Reagents required:—

(1) **For actual stain:**—Any form of violet stain, either methyl violet, gentian violet or crystal violet may be used.

Methyl violet:—

Methyl violet	0·5 to 1 gram.
Distilled water	100 c.c.

Filter.

(2) **For mordanting:**—Iodine solution is employed and is prepared as follows:—

Iodine	1 gram.
Potassium iodide	2 grams.
Distilled water	100 c.c.

(Gram's iodine is three times weaker than the above).

(3) **For decolorisation:**—Absolute alcohol, acetone, or aniline-xylol may be used.

(4) **For counter-stain:**—Saffranin, neutral red or weak carbol-fuchsin may be used.

Saffranin:—

Saffranin	0·5 gram.
Distilled water	100 c.c.

Filter.

Neutral red:—

Neutral red	1 gram.
Distilled water	1,000 c.c.
Acetic acid, 1 per cent	2 c.c.

Carbol-fuchsin:—

Ziehl-Neelsen's carbol-fuschin (P. 21) ...	1 c.c.
Distilled water	20 c.c.

Technique (Jensen's modification)

1. **Preparation of the film:**—A thin uniform film is absolutely essential. Because, if this is uneven, *i.e.*, thick in one part and thin in another, it is practically impossible to obtain a good result. When the thin parts are correctly treated with alcohol, the thick parts are not sufficiently decolourised; while again, when the thick parts are correctly treated the thin portion will be over-decolourised.

2. **Fixation of the film:**—The film is dried in air and fixed by rapidly passing the slide, film side up, two or three times over the flame of a Bunsen burner holding the slide with the thumb and index finger. The slide is then allowed to cool. The object of this *fixing*, is to coagulate the albuminous material whereby the film adheres better to the glass and thus may not be detached in the subsequent processes.

Note:—A film should never be heated over the flame until it is thoroughly dried up, because heating a moist film will produce distortion of structure.

3. **Staining:**—The slide is stained with methyl violet which may be filtered before use. To prevent the stain flowing over the slide, two thick lines may be drawn with a grease pencil, one on either side of the area to be stained. The stain is allowed to act for ½ to 1 minute.

4. **Mordanting:**—The stain is *washed off* with iodine solution. It is to be remembered carefully that methyl violet should not be thrown away but rather displaced by flooding it off with iodine solution and, being sure that all the violet stain has been removed, the film is covered with the mordant and left for 1 to 2 minutes.

5. **Decolorisation with absolute alcohol:**—The film is now treated with absolute alcohol, drop by drop (about 6 drops at a time). After the slide is rocked and rinsed with alcohol for a few seconds, it is poured off and replaced by fresh alcohol and the whole washing process is thus repeated several times until the violet colour ceases to come off.

6. **Counter-staining:**—The film is then counter-stained with saffranin solution for 1 to 2 minutes.

7. **Washing:**—The slide is then washed in tap water and dried in air or between blotting papers. It is then ready for examination under oil-immersion lens.

Note:—Washing is not done until the completion of (6).

Precautions against failures:—

(i) Accurate timing and for this a watch is essential.
(ii) Undivided attention should be paid.
(iii) One slide only should be stained at a time.
(iv) Film must be thin and uniform.
(v) Methyl violet should always be filtered.
(vi) Careful washing with iodine solution should be done.
(vii) Judicious use of alcohol for decolorisation.
(viii) Tap water should be used last of all.

Principle of the stain:—When an organism is stained with the violet stain and is subsequently treated with iodine solution, the latter increases the colouring affinity of the stain to the micro-organism. Under such a condition, certain organisms resist subsequent decolorisation with alcohol, whereas others can not. The organisms which resist decolorisation and therefore retain the violet stain are termed Gram-positive. The organisms which are decolourised and therefore cannot retain the violet stain are termed Gram-negative. These latter have to be shown up in a preparation by a contrast counter-stain such as weak carbol-fuchsin, neutral red or saffranin. Thus the method divides all bacteria into two classes (i) Gram-positive and (ii) Gram-negative.

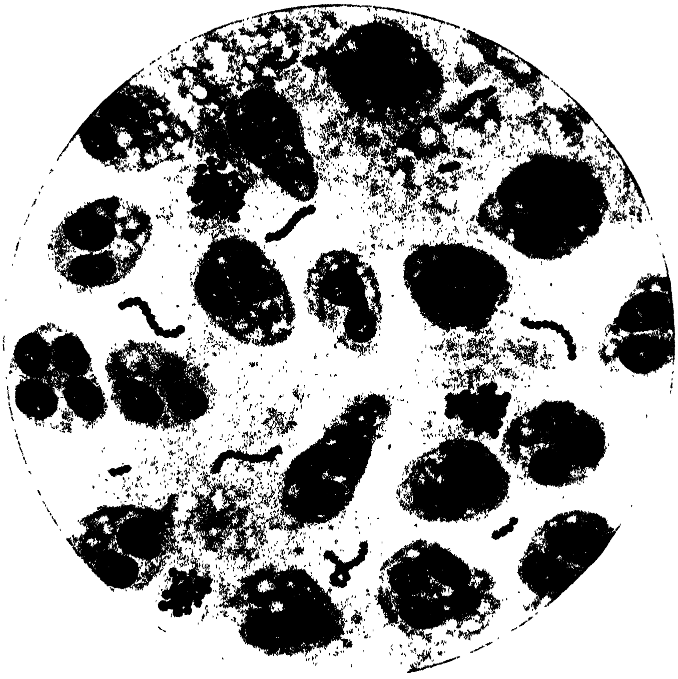


Fig. 21.

Smear preparation of pus stained by Gram's method, showing the Gram-positive character (violet colour) of Streptococci and Staphylococci and Gram-negative character (pink colour) of pus cells.

Reasons for the retention of the violet stain by the Gram-positive organisms:—

1. **Physical theory:**—It is supposed to be due to the difference in the permeability of the cell membrane of different micro-organisms.

2. **Chemical theory:**—Gram-positiveness is due to the presence of some particular chemical compound, probably a neucleoprotein.

3. **Intermediate theory:**—A compound formed by the interaction of the iodine and a constituent of the dye (violet) is firmly linked to the bacterial protoplasm imparting to it, its Gram-positiveness. The colour is therefore not removed by subsequent treatment with alcohol. But in the negative ones, it is readily decolourised.

Appearances with Gram's stain:—Gram-positive organisms and fibrin are stained a deep violet colour. Gram-negative organisms, pus cells and tissue cells are stained pink with the counter-stain. (Fig. 21).

Examples:—Practically all pathogenic cocci are Gram-positive except the Neisseria group (N. gonorrhœæ, N. meningitidis, N. catarrhalis, etc.). All pathogenic bacilli are Gram-negative except the Spore-bearing, Acid-fast and Diphtheria groups.

TABLE SHOWING THE REACTIONS OF IMPORTANT BACTERIA TO GRAM'S STAIN

COCCI

Gram-positive	*Gram-negative*
Streptococci Staphylococci Diplococcus pneumoniæ (Pneumococcus) Gaffkya tetragena (Micrococcus tetragenus) Sarcinæ	Neisseria group e.g., N. gonorrhœæ (Gonococcus) N. meningitidis (Meningococcus) N. catarrhalis (Micrococcus catarrhalis), etc.

BACILLI

Acid-fast group (Mycobacterium), e.g. M. tuberculosis (B. tuberculosis) M. lepræ (B. lepræ) M. smegmatis (B. smegma) etc.	Coli-typhoid-dysenteriæ group: Eberthella group, e.g. E. typhi (B. typhosus) E. dysenteriæ (B. dysenteriæ, Shiga) E. paradysenteriæ (B. dysenteriæ, Flexner, Strong and Y)
Diphtheria group (Corynebacterium), e.g. C. diphtheriæ (B. diphtheriæ) C. hofmani (B. hofmani) C. xerosis (B. xerosis) etc.	Escherichia group, e.g. E. coli (B. coli communis) etc.
Spore-bearing group Aerobic (Bacillaceæ), e.g. B. anthracis B. subtilis etc.	Salmonella group, e.g. S. paratyphi (B. paratyphosus A) S. schottmülleri (B. paratyphosus B)

BACILLI (Continued)

Gram-positive

Anærobic (Clostridia), e.g.
  Cl. tetani (B. tetanus)
  Cl. botulinum (B. botulinus)
  Cl. welchii (B. welchii)
  Cl. sporogenes (B. sporogenes)
  etc.

Gram-negative

S. ærtrycke (B. ærtrycke)

S. enteritidis (B. enteritidis, Gärtner)

S. morgani (B. morgan No. 1)
  etc.

Pasteurella group, e.g.
  P. pestis (B. pestis)
  etc.

Pfeifferella group, e.g.
  Pf. mallei (B. mallei; Glanders bacillus)

Hæmophilic group, e.g.

  H. influenzæ (B. influenza, Pfeiffer's bacillus)

  H. pertussis (B. pertussis; Bordet-Gengou-bacillus of whooping cough)

  H. conjunctivitidis (Koch-Weeks bacillus of acute infectious conjunctivitis)

  H. lacunatus (Morax-Axenfeld bacillus of subacute infectious conjunctivitis or angular conjunctivitis)

  H. ducreyii (Ducrey's bacillus of soft chancre)

Klebsiella group, e.g.
  K. pneumoniæ (Friedländer's pneumobacillus)
  etc.

Dialister pneumosintes (Bacterium pneumosintes)

Brucella melitensis (B. melitensis; Alkaligenes melitensis; or bacillus of Malta fever)

Pseudomonas æruginosa (B. pyocyaneus)

SPIRILLÆ

Vibrio, e.g.
  V. comma (V. choleræ), etc.

Spirochætes, e.g.
  Treponema recurrentis
  Treponema pallidum
  Leptospira ictero-hæmorrhagica
    etc.

II. ACID-FAST STAIN

Certain bacteria possess the characteristic property of retaining the red colour of fuchsin stain after treatment with dilute mineral acids. Hence they are termed "*Acid-fast.*"

Examples:—

A. Pathogenic to man.

1. Mycobacterium tuberculosis (B. tuberculosis).
2. Mycobacterium lepræ (B. lepræ).

B. Non-pathogenic to man (Saprophytes).

3. Mycobacterium smegmatis (B. smegma).
4. Mycobacterium butyricum (The Petri-Rabinowitsch butter bacillus).
5. Mycobacterium phlœi (Mœller's timothy-grass bacillus).

Reagents required:—

(1) **For the actual stain:**—Ziehl-Neelsen's carbol-fuchsin is used and prepared as follows:—

Saturated alcoholic solution of basic fuchsin ...	1 part.
Carbolic acid solution (1 in 20) ...	9 parts.

Note:—Basic fuchsin is the stain and carbolic acid is the mordant.

(2) **For decolorisation:**—Mineral acids are used, such as, 20 per cent. nitric acid, 25 per cent. sulphuric acid, 3 to 4 per cent. hydrochloric acid, or Czaplewsky's solution.

Czaplewsky's Solution:—

Sodium chloride ...	2·5 grams.
Acid hydrochloric ...	20·5 c.c.
Ethyl alcohol (95%) ...	400 c.c.
Distilled water ...	100 c.c.

(3) **For counter-stain:**—Methylene blue, picric acid or malachite green may be employed.

Carbol-Methylene Blue:—

Saturated alcoholic solution of methylene blue ...	5 c.c.
Carbolic acid solution (1 in 20) ...	95 c.c.

Picric acid—Saturated watery solution.

Malachite green—1 per cent. solution.

ZIEHL-NEELSEN'S TECHNIQUE

(1) **Preparation and fixation of the film:**—A portion of the mucopurulent material is taken on a slide and a smear is made with another slide or with a platinum loop and dried in air. The dry film is then fixed over a flame.

(2) **Staining:**—Some hot carbol-fuchsin is poured on the slide or the slide with the stain is heated to steaming, after which, the stain is allowed to act for 5 to 7 minutes.

Precautions:—(i) The stain should not be allowed to boil or dry up on the slide, otherwise dirty precipitates will be formed on the film.

(ii) A mark with a grease pencil about half an inch from the end prevents the stain from running over the entire surface of the slide.

(3) **Decolorisation:**—The excess of stain is washed in water and the slide is then decolourised in sulphuric acid till a faint pink colour is obtained. The acid is washed off well in tap water.

Precautions:—(a) The acid solution should not be kept on the slide for more than 10 seconds at a time. The acid may be changed two or three times to bring out the desired pink colour. It is convenient to have a bath containing this fluid into which the slide may be dipped in and taken out. In this step when the amount of decolorisation is observed, the slide should be washed in running tap water to remove the excess of acid. It is in this way only the correct decolorisation is to be obtained.

(b) During decolorisation the opposite side of the slide should be washed clean so that no carbol-fuchsin stain sticks on it, otherwise it may give a false appearance of the stain on the smear, resulting in over-use of the decolourising agent.

(4) **Counter-stain:**—After washing the slide thoroughly in tap water, it is stained with methylene blue for 1 to 2 minutes.

(5) **Washing:**—It is then washed in tap water, dried and examined under oil-immersion lens.

Precautions against failure:—

(i) Selection of material. It is always advisable to pick up a mucopurulent, caseous material, which contains the largest number of bacilli.

(ii) Use a uniform smear.

(iii) Never heat too much to produce boiling or charring.

(iv) Pay undivided attention during decolorisation.

(v) Decolourise in stages.

Modifications of the technique:—

(a) **Use of 95% Alcohol:**—After the use of dilute acids, decolorisation can further be effected by alcohol, particularly to differentiate M. tuberculosis (alcohol-fast) from M. smegmatis (not alcohol-fast). The latter retains the colour of carbol-fuchsin when treated with dilute acids (acid-fast) but loses it when treated with alcohol in addition (not alcohol-fast). Hence acid-alcohol technique should specially be used, in examining a sample of urine for the presence of M. tuberculosis, in order to eliminate M. smegmatis.

(b) **Combined acid-alcohol technique:**—Instead of using acid and alcohol separately for decolorisation, the method may be combined by using (i) 1 per cent. hydrochloric acid in absolute alcohol or (ii) Czaplewsky's solution (P. 21).

(c) **Counter-staining** may be done with a saturated aqueous solution of picric acid. This gives a very clean field and clear contrast.

Appearance with acid-fast stain:—Acid-fast organisms are stained bright red whilst tissue cells and other organisms are stained blue, yellow or green according to the nature of the counter-stain used (Fig. 22).

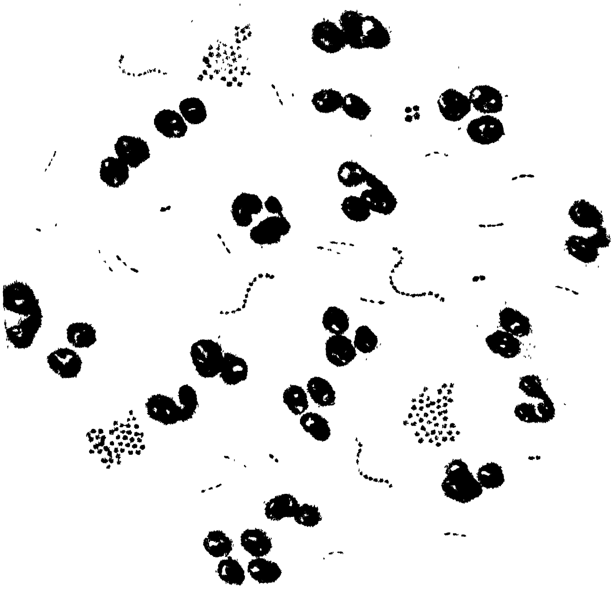


Fig. 22.

Smear preparation of sputum stained by Ziehl-Neelsen's method, showing the acid-fast character (red colour) of M. tuberculosis and non-acid-fast character (greenish blue) of others. Malachite green is used as the counterstain.

Principle of the stain:—Owing to the presence of a fatty envelope, these organisms resist penetration of the aniline dyes, and are therefore not stained by the simple routine stains. Hence they require special methods of staining. A powerful stain, such as basic fuchsin, is used and to it carbolic acid is added as a mordant to increase its fastness and, in order to intensify further its penetrating power, heat is also applied. Since these organisms are resistant to dye penetration, they also tend to resist decolorisation by mineral acids (acid-fast) as well as by alcohol (alcohol-fast). The degree of fastness serves to differentiate the individual members of the groups. As for example:—

(i) Tubercle bacillus is both acid and alcohol-fast.

(ii) Smegma bacillus is only acid-fast but not alcohol-fast.

(iii) Lepra bacillus is acid-fast to a less degree and is also less alcohol-fast.

SPECIAL STAINS

SPECIAL STAINS FOR META-CHROMATIC GRANULES OF CORYNEBACTERIUM DIPHTHERIÆ

I. Neisser's stain (original method)

Reagents Required:—

1. *Neisser's methylene blue:—*

Methyene blue	0·1 gram.
Absolute alcohol	2 c.c.
Glacial acetic acid	5 c.c.
Distilled water	95 c.c.

Dissolve the methylene blue in absolute alcohol and add it to the acetic acid-water mixture. Filter.

2. *(a) Bismarck brown solution:—*

Bismarck brown	0·2 gram.
Water (boiling)	100 c.c.

Dissolve the stain in boiling water and filter.

or (b) Chrysoidin solution:—

Chrysoidin	1 gram.
Distilled water	300 c.c.

Dissolve by gentle heat and filter.

Technique:—

(i) Prepare and fix films as usual.
(ii) Pour the Neisser's stain on the film and allow it to act for a minute.
(iii) Wash with water.
(iv) Pour on the Bismarck brown solution or chrysoidin solution and allow the stain to act for 15 to 30 seconds.
(v) Wash off with water and dry.

Remark:—The protoplasm of the bacillus is stained yellowish while the granules appear as dark blue dots at either end. To obtain good results, it is necessary that cultures be not more than 18 hours old.

II. Pugh's stain

Stain:—

Toluidin blue	2 grams.
Absolute alcohol	20 c.c.
Glacial acetic acid	50 c.c.
Distilled water	950 c.c.

Technique:—

(i) Fix the smear by heat as usual.
(ii) Flood the film with the stain and heat to steaming. Allow the stain to act for one to three minutes.
(iii) Wash in water, dry and examine under oil immersion lens.

Remark:—This is a very useful and rapid single stain. The protoplasm of the bacilli is stained light blue and the granules are coloured reddish purple.

III. Ponder's stain

Stain:—

Toluidin blue (Grübler)	... 0·02 gram.
Glacial acetic acid	... 1 c.c.
Absolute alcohol	... 2 c.c.
Distilled water to make	... 100 c.c.

Technique:—

(i) Make a film on a cover glass and fix it in the usual way.

(ii) Spread the stain on the film, turn over the cover-slip and mount as a wet preparation.

Remark:—The metachromatic granules come out with great intensity and the bacillary body is stained a lighter colour.

IV. Bie's stain

Stain:—

Methylene blue	... 2 grams.
Dahlia	... 0·25 gram.
Absolute alcohol	... 20 c.c.
Glacial acetic acid	... 50 c.c.
Distilled water	... 950 c.c.

The stains are dissolved in alcohol which is then added to the mixture of acetic acid and water.

Technique:—

(i) Make a film on a cover-glass and fix it in the usual way.

(ii) Put a drop of the stain on the slide and mount cover-glass on it. Remove the excess of the stain by soaking it in blotting paper.

(iii) Examine the preparation under oil immersion lens, without delay.

Remark:—The preparation is not permanent and fades in a short time. Diphtheria bacilli are seen with pale blue protoplasm and deep blue granules; other organisms are only feebly stained.

V. Stain of von Staltenberg, H (The journal of laboratory and clinical medicine. Vol. XIII, No. 7, April, 1928.)

Stain:—

Malachite green	... 0·25 gram.
Toluidin blue	... 0·05 gram.
Hæmatoxylin	... 0·01 gram.
Distilled water	... 100 c.c.
Acetic acid	... 3 c.c.
Absolute alcohol	... 3 c.c.

Dissolve malachite green and toluidin blue in distilled water and hæmatoxylin in absolute alcohol; then add acetic acid. Filter.

Technique:—

(i) Fix the film in the usual way.

(ii) Pour the solution on the slide and allow it to act for 2 to 5 minutes.

(iii) Wash in tap water, dry and examine under oil immersion lens.

Remark:—The granules are stained red or purple and the bodies are green. (Fig. 23).

VI. Gutstein and Neisser's stain (Azure II stain)

(Journal of laboratory and clinical medicine Vol. XVII, No. 5, February, 1932.)

Reagents Required:—

1. Stain—1 per cent. acqueous solution of Azure II.
2. Decolourising agent—0·5 per cent. acetic acid or aqueous solution of chrysalinine.

Technique:—

(i) Stain 2 to 3 minutes with 1 per cent. acqueous Azure II solution.

(ii) Wash in tap water.

(iii) Differentiate with 0·5 per cent. acetic acid for one to three seconds.

Metachromatic granules are stained reddish-violet, while the bodies are stained blue. (Fig. 24.)

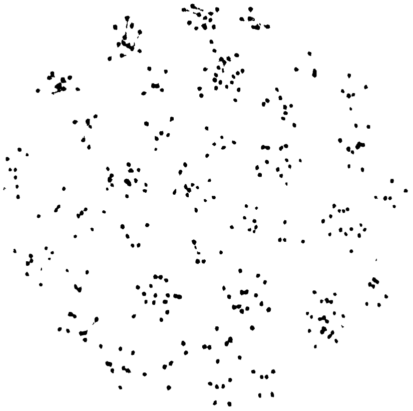


Fig. 23.
Von Staltenbergh stain of C. diphtheriæ.

or (i) Stain 2 to 3 minutes with 1 per cent. aqueous Azure II solution.

(ii) Wash in tap water.

(iii) Wash with aqueous chrysalinine.

Metachromatic granules are stained black and the bodies of the bacilli are yellow.

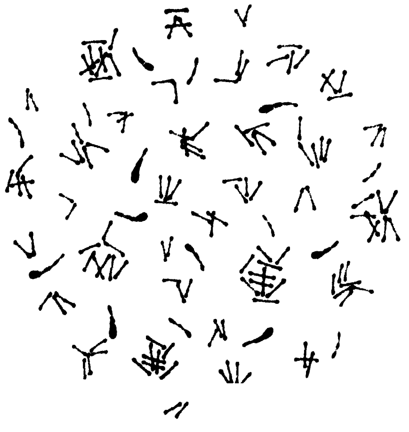


Fig. 24.
Azure II stain of C. diphtheriæ; some involution forms are shown.

STAINING OF CAPSULES:—

Capsule of a bacterium may be demonstrated either by relief methods, *i.e.*, the capsules are left unstained on a coloured background; or by staining of the capsular substance.

(a) RELIEF METHOD:—

I. Benian's congo-red method

Reagents Required:—

1. Congo red solution (2 per cent.).

2. Acid-alcohol (1 per cent. hydrochloric acid in absolute alcohol).
3. Methyl violet.

Technique:—

(i) Take a drop of congo red solution and a loopful of the material on a clean glass slide, mix thoroughly and spread out as a thin film; dry.

(ii) Flood the slide with acid alcohol and allow to act for one minute. This turns the film blue.

(iii) When dry, counterstain with methyl violet solution for 5 minutes, heating to steaming.

(iv) Wash well, dry and examine under oil immersion lens.

Remark:—Capsule stands out as an unstained halo and the protoplasm of the bacteria takes a violet stain. The film may be examined before counterstaining when the capsule appears as a silvery white halo against a dark blue background.

II. Indian ink method of Burri:—This does not give a very satisfactory result.

(b) STAINING OF THE CAPSULAR SUBSTANCE

I. Hiss's method

Reagents required:—

(1) Saturated alcoholic solution of basic fuchsin ... 1 part.
Distilled water ... ... 19 parts.

(2) 20 per cent. aqueous copper sulphate solution.

Technique:—

(i) Fix a thin film on a slide in the usual manner.

(ii) Place a few drops of solution (1) on the film and heat to steaming. Allow the stain to act for 30 seconds.

(iii) Wash off the stain with a liberal amount of solution (2).

Note:—In order to avoid too much deposit of copper sulphate crystals on the film, the solution should be used in excess and poured on the slide until the latter is cool.

(iv) Dry between blotting papers (do not wash) and mount, if necessary, in Canada balsam.

Note:—Water should not be applied at any stage of the procedure.

Remark:—The method is easily applied and gives excellent results. By this method the capsule appears as a faint blue halo around a dark purple cell body. (Fig. 25.)

II. Richard Muir's method

Reagents required:—

1. *Stain.*

Strong carbol-fuchsin (Ziehl-Neelsen's.)

2. *Special mordant.*

Saturated solution of corrosive sublimate	2 parts.
20 per cent. solution of tannic acid	2 parts.
Saturated solution of potassium alum	5 parts.

3. *Counterstain.*

Loeffler's methylene blue.

Fig. 25.

Capsule of D. pneumoniæ in a smear preparation from heart's blood of mice, stained by Hiss's method.

Technique:—

(i) Make a thin film and fix by heat as usual.

(ii) Stain with filtered carbol-fuchsin for half to one minute, the preparation being gently heated to steaming.

(iii) Wash slightly with rectified spirit and then well in water.

(iv) Pour on the mordant, and allow it to act for 30 seconds.

(v) Wash well in water.

(vi) Treat with methylated spirit for about a minute. The time however varies with the preparation and is found by experience. Usually 30 to 40 seconds are sufficient. After treating with spirit, the film should be pale red in appearance.

(vii) Wash well in water.

(viii) Counterstain with Loeffler's methylene blue for half to one minute.

(ix) Dehydrate in absolute alcohol, clear in xylol and mount in balsam.

Remark:—The bacteria are stained deep crimson while the capsules are of blue tint. Richard Muir's method gives beautiful results but is much more difficult for the beginner and is not recommended for routine work.

STAINING OF SPORES:—When spore-bearing bacilli are stained by ordinary methods (with watery solutions of basic aniline dyes), the body of the bacillus is deeply coloured whereas the spores remain unstained and appear as clear areas. This seems to be due to the fact that the spores have a resisting envelope which prevents the entrance of the staining agent. They may however be stained by using some special methods which allow the penetration of the dye. Once stained, they retain the colour with considerable tenacity like the tubercle bacilli; the resisting envelope preventing the full action of the decolourising agent.

I. Modified Ziehl-Neelsen's method:—

Reagents required:—

1. Ziehl-Neelsen's carbol-fuchsin (P. 21).
2. Sulphuric acid (0·5 per cent.) or methylated spirit.
3. Loeffler's methylene blue.

Technique:—

(i) Make a thin emulsion on the slide from an old culture kept at room temperature; dry, and fix by passing once or twice over the flame.

(ii) Stain with Ziehl-Neelsen's carbol-fuchsin for 5 to 10 minutes, heating from time to time until steam rises.

(iii) Wash in tap water.

(iv) Decolourise with (a) 0·5 per cent. sulphuric acid, if the acid be stronger than 1 per cent., the spores of many bacilli are readily decolourised); or (b) methylated spirit.

(v) Wash in water.

Decolorisation is observed from time to time under the 1/6 in. objective. When the slide is nearly colourless, it is examined. If the spores, as well as the bacilli, retain the red colour, the preparation must be further decolourised. If the spores alone are stained, it is counter-stained.

(vi) Counter-stain with Loeffler's methylene blue for two minutes.

(vii) Wash in water, blot and dry; mount in balsam.

Remark:—The spores are stained bright red and the protoplasm of the bacilli blue. (Fig. 26).

II. Möller's method:—

In this method, the films before being stained are treated with chloroform for 2 minutes, well washed and then placed in a 5 per cent. solution of chromic acid for ½ to 2 minutes, after which they are again well washed in

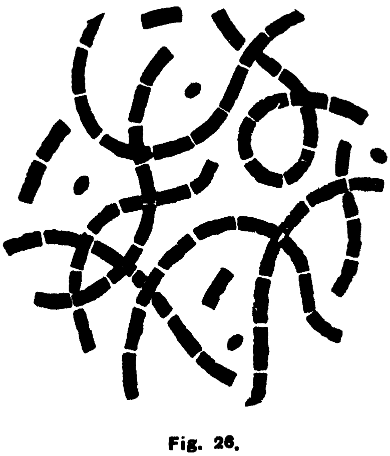


Fig. 26.

Staining of spores of B. anthracis by modified Ziehl-Neelsen's method.

water. Then they are stained and decolourised as above. This method is much more satisfactory as it makes the spores easily permeable to the staining reagent.

III. Schaeffer and Fulton's method (The journal of laboratory and clinical medicine Vol. XVII No. 9, June 1933). This is a very simple method of staining endospores.

Reagents required:—

1. *Stain:*—5 per cent. aqueous solution of malachite green. Dissolve, allow to stand for 30 minutes, and filter.
2. *Counter-stain:*—0·5 per cent. aqueous solution of saffranin.

Technique:—

- (i) Prepare films and fix as usual.
- (ii) Flood with malachite green and heat to steaming.
- (iii) Wash in tap water for 40 seconds.
- (iv) Apply saffranin and allow the stain to act for 30 seconds.
- (v) Wash, blot, dry and examine.

Endospores are stained green and the protoplasm of the bacilli red. (Fig. 27).

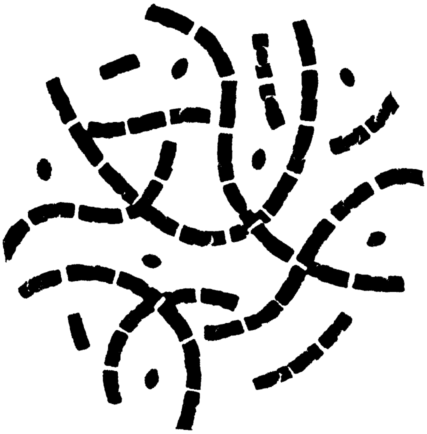


Fig. 27.
Staining of spores of B. anthracis by Schaeffer and Fulton's method.

STAINING OF FLAGELLA:—

Many organisms possess delicate flagella which are not visible by the ordinary methods of staining. They are also not visible when the motility of the organism is examined ordinarily in a hanging-drop preparation. They may, however, be seen when examined by dark ground illumination. To demonstrate the presence of flagella, special stains are therefore necessary. In this connection, it should be noted that the staining of flagella is the most difficult of the bacteriological procedures. Success depends on rigorous cleanliness of slides, freshly prepared solution, a fresh culture and the growth sufficiently diluted to obtain the organisms in an isolated condition.

Preparation of films:—Take a perfectly clean (grease free) slide and place a drop of pure distilled water on its centre. A very small portion of the growth is taken, on the point of a platinum needle from a young culture on agar (10 to 18 hours' cultures) and held in contact with the water for a little while. The point of the needle may be moved gently in the water. This allows the bacteria to swim out in the fluid producing a very thin emulsion. The amount will be such as will cause the faintest turbidity with water. It is then spread out and allowed to dry in the air.

Or, a thin bacterial emulsion may be made in a test tube containing about 1 to 2 c.c. of distilled water and a loopful of this may be spread on a slide and dried as before.

Muir's modification of Pitfield's method:—

Reagents required:—

1. *Mordant:—*

Tannic acid, 10 per cent. watery solution (filtered)	...	10 c.c.
Corrosive sublimate, saturated watery solution	...	5 c.c.
Alum, saturated watery solution	...	5 c.c.
Ziehl-Neelsen's carbol-fuchsin stain	...	5 c.c.

Mix thoroughly and centrifugalise; remove supernatant clear coloured fluid with a pipette and transfer to a clean bottle. This will keep for two or three weeks.

2. *The stain* (freshly prepared).

Alum, saturated watery solution (filtered)	...	10 c.c.
Gentian violet, saturated alcoholic solution	...	2 c.c.

Technique:—

(i) Flood the prepared slide with the mordant; heat to steaming for 1 minute and then let it stain for 10 minutes. Wash well in a stream of running water for 2 minutes. Dry carefully over a flame.

(ii) Pour some stain, steam gently for 1 minute; wash well in water, dry; mount in a drop of Canada balsam.

COMPOUND STAINS

THE ROMANOWSKY STAINS:—The peculiar property of these stains is that a compound is formed due to the interaction of methylene blue and eosin. This imparts a reddish purple colour to the chromatin of the various micro-organisms. Before the methylene blue is mixed with eosin, it is "ripened," either by age or by heating with sodium carbonate. It is then mixed with eosin in varying proportions and the precipitate which forms, is dried, washed in distilled water and finally dissolved in acetone-free methyl alcohol. Each modification of Romanowsky stain differs mainly in the (i) relative proportions of methylene blue and eosin and (ii) the method of ripening methylene blue before it is allowed to combine with eosin. These stains, when not used, should be kept in a dark place as they deteriorate on exposure to the tropical sun. The alcoholic preparations of the stains are stocked in a well-stoppered bottle otherwise they become too concentrated by the evaporation of alcohol and thus stain deeply or form a precipitate on the film.

LEISHMAN'S OR WRIGHT'S STAIN:—

Preparation of the stain:—

Solution A:—

Methylene blue (Grübler)	...	1 gram.
Sodium bicarbonate	...	0·5 gram.
Distilled water	...	100 c.c.

The mixture may be heated in a Koch's steam steriliser or a water-bath for an hour to make it polychrome. After heating, the mixture is allowed to cool by placing the flask in cold water and is then filtered to remove all precipitates which might form in it. It should, when cold, have a deep purple red colour when viewed in a thin layer by transmitted yellowish artificial light. It does not show this colour so long it remains hot.

In our tropical climate, an excellent result may be obtained by exposing the mixture in a flask or a bottle to the sun, successively for some days, till the blue colour changes into purple. During this time, the mixture should be frequently shaken. Usually about 10 to 15 days are necessary but the time of exposure depends on the strength of the sun's rays. Too long an exposure causes a precipitate and spoils the solution.

Solution B:—

Eosin, extra B.A. (Grübler)	...	0·5 gram.
Distilled water	...	500 c.c.

In a large open vessel, add slowly solution B to solution A, stirring constantly. The mixture becomes purple and a scum appears on the surface with a bright metallic lustre. There is also formed a finely granular precipitate at the bottom. Filter and collect the residue on a filter paper and dry in the incubator. 0·3 gram of this dry powder is dissolved in 100 c.c. of acetone-free methyl alcohol. To effect the solution of the powder, it is ground in a mortar with successive portions of methyl alcohol, decanting the clear fluid from time to time. The process is repeated several times until all the residue (undissolved stain) goes into the solution. The stain is improved by keeping it in the incubator for 2 to 3 days, before use.

Uses:—(1) To stain blood film for cytological examination and detection of parasites.

(2) To stain certain bacteria such as Pasteurella pestis.

Method of staining blood film:—

(i) Allow the film to dry in air.

Note:—It must never be fixed by heat.

(ii) Cover the film with undiluted stain and allow it to act for half a minute (the methyl alcohol in the stain fixes the film).

Note:—The slide should be covered with a bell-jar, as by this means evaporation of alcohol is prevented and precipitation of the stain on the film is avoided.

(iii) Add double the quantity of distilled water and mix it thoroughly by alternately sucking in and out with a pipette or by gentle shaking. The addition of water and mixing should be done carefully so that overflowing from the slide is prevented. Allow the diluted stain to act for 10 minutes. If it is kept longer, the stain becomes of a dirty colour.

Note:—Although it is the usual belief to dilute the stain with distilled water, excellent result may be obtained with ordinary tap water. A very slight degree of alkalinity in the tap water makes the latter an admirable diluting fluid.

(iv) Wash the slide in running tap water or distilled water. Let the film be covered with water for a minute to wash off the excess of blue colour. Dry and examine under oil immersion lens.

Note:—The stain should never be thrown away and then the slide washed in water but rather the water should displace the stain.

Disadvantages of Leishman's stain:—As it is an alcoholic preparation, the volatility of alcohol renders it unsuitable to keep for a sufficient length of time either in the stock bottle or in the staining films. It is for this reason that special attention should be paid when Leishman's stain is used.

GIEMSA'S STAIN:—This stain consists of a number of compounds which are designated as Azur II and Azur II-eosin and are formed by the combination of varying proportions of methylene blue and eosin. They may be purchased ready-made from Grübler. The stain is prepared as follows:—

Azur II-eosin ...	3 grams.
Azur II ...	0·8 gram.
Glycerin (Merck, chemically pure) ...	250 grams.
Methyl alcohol (Kahlbaum I) ...	250 grams.

This forms the stock solution. Before actual use, 1 part of the stock solution should be diluted with 10 to 15 parts of distilled water.

Uses:—(1) To stain blood films for cells or parasites.

(2) To stain spirochætes, the process involving prolonged staining.

Staining of blood films:—

(i) Fix the blood film with absolute alcohol for a couple of minutes.

(ii) Stain with Giemsa's stain (diluted 1 in 10 with distilled water) and allow it to act for 15 minutes.

(iii) Wash in distilled water.

(iv) Dry in air and examine under oil immersion lens.

Advantages:—The stain does not become concentrated on keeping and can be left on the film longer than the specified time without any untoward result.

Prolonged staining for spirochætes:—

(i) Fix the preparation in absolute alcohol for 3 to 5 minutes.

(ii) Immerse the slide in the diluted stain overnight. For this purpose the stain may be kept in a small covered glass jar and the slide placed in it in a standing position.

(iii) Wash the slide in distilled water, dry in air and examine under oil immersion lens.

The following is a very good *formula for the dilution of Giemsa's stain* and gives excellent results:—

Stock Giemsa's solution ...	2·5 c.c.
Methyl alcohol ...	2·5 c.c.
Distilled water ...	100 c.c.
0·5 per cent. sodium carbonate solution ...	5 drops.

JENNER'S STAIN:—It is prepared by mixing equal parts of 1·2 per cent. aqueous solution of eosin (Grübler, water-soluble) and 1 per cent. aqueous solution of methylene blue (Grübler) and allowing it to stand for 24 hours. It is then filtered and the residue is dried at 55°C. This is redissolved in distilled water, filtered and the residue dried again. This dried powder is the stain and it should be noted that in its preparation no alkali has been used. For use 0·5 gram of the powder is dissolved in 100 c.c. of acetone-free methyl alcohol and a few drops of the stain are poured on the dried, unfixed film. The stain is allowed to act for 1 to 3 minutes, washed in distilled water till it becomes pink and then dried in air or between blotting papers. The slide is now ready for examining under oil immersion lens.

VITAL STAINING

I. ***Intra-vitam staining:***—This staining method is employed mainly for the study of the reticulo-endothelial system of cells. The entire staining process goes on inside the body of an animal which is alive and thus the activity of a living cell forms the basis of this method. When a certain dye is injected into the tissues of a living animal, the greater part of it is usually excreted through the urine and fæces but a small part is retained and stored up as fine particles inside the cytoplasm of the reticulo-endothelial cells. This special group of cells can thus be readily recognised under the microscope. The dye stuffs which are commonly used for the purpose are Indian ink, carmine, trypan blue, and pyrrol blue, although a large number of others may also be used. Of these stains, Indian ink is somewhat coarse and very delicate staining cannot be obtained with it. The best way to use it, is to take any sample of indelible ink, mix it with normal saline and centrifuge the mixture to allow the coarser particles to settle at the bottom. The supernatant fluid is then injected daily into the animal, either intraperitoneally or intravenously. The dose should be small, not exceeding 1 c.c. The injections should be repeated 6 to 8 times, when the animal will assume a somewhat blackish colour. It may then be killed and the tissues examined histologically.

As for the other staining reagents, a 1 per cent. solution in normal saline is commonly employed. The dose should be approximately 1 c.c. per 20 grams of body weight of the animal. If the quantity becomes too bulky, a 2 per cent. solution may also be used. The injections may be made subcutaneously, intraperitoneally or intravenously. To get satisfactory staining, repeated intraperitoneal injections or repeated small intravenous injections are necessary. Usually, about 6 to 8 injections are required. These dyes, when used in such low strengths, do not cause much harm to the animal. At first it is intensely stained all over its body and the urine becomes loaded with the pigment, but later the colour may rapidly fade owing to quick elimination. The animal should be killed after the required number of injections and its tissues fixed in 10 per cent. formol-saline. Sections may be prepared either by the freezing or by the paraffin embedding method. The nuclei of the cells may be stained by the ordinary nuclear stains preferably by alum hæmatoxyline, and counter-stains should be avoided to prevent confusion.

In this way it may be found that the pigment has been most markedly stored up by the Kupffer's cells of the liver (Fig. 28), the mononuclear cells of the splenic pulp and lymphatic glands and also the reticulo-endothelial cells of the bone-marrow. Such cells in other parts of the body also take the colour though not so markedly as the former.

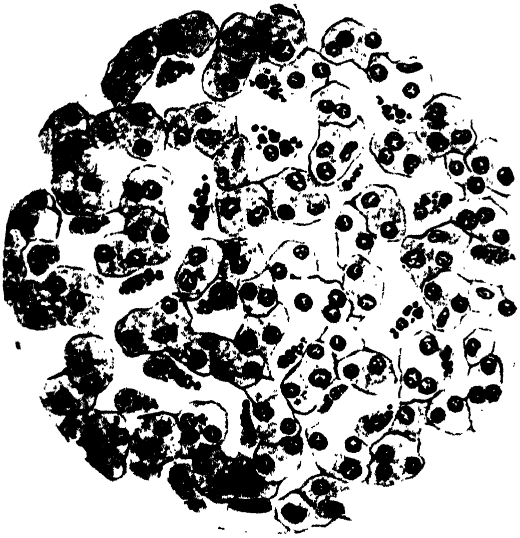


Fig. 28.

Section of a liver showing intra-vitam staining of Kupffer cells with Indian ink.

II. ***Supra-vitam staining:***—This is a process of staining which takes place in cells which are outside the animal body but which are still alive for some time. The commonest example is the staining of the reticulocytes of blood with brilliant-cresyl-blue. It may also be used with other stains to study the phagocytic activity of leucocytes and other tissue cells.

For blood films and bacteria:—Stains used are neutral red, methylene blue, brilliant cresyl blue and Janus green. These dyes are kept in stock, as saturated alcoholic solutions, in glass-stoppered bottles. The stains used for different materials are as follows:—

For bacteria—Neutral red or methylene blue.
For reticulocytes—Brilliant cresyl blue.
For polymorphonuclear cells—Neutral red.
For mitochondria—Janus green.

Small drops of blood, cell suspensions or bacteria are placed on specially prepared slides and then incubated for a few minutes or examined on the warm stage. The slide may be covered with a cover-slip and sealed with vaseline.

How to prepare slides:—30 to 50 drop [illegible] required dye are added to 10 c.c. of absolute alcohol and the soluti [illegible] ured over a clean warm slide. The excess is thrown away and the s [illegible] ried in the vertical position.

CHAPTER III

GENERAL BIOLOGY OF BACTERIA

CONDITIONS FOR GROWTH:—There are five main factors which have a marked influence on the promotion of bacterial growth. These are (1) food supply, (2) gaseous environment, (3) moisture, (4) temperature and (5) light.

1. ***Food supply:***—All bacteria consist of carbon, hydrogen, oxygen, nitrogen, phosphorus, sulphur and inorganic salts. Hence these must be supplied to them for their growth. When we try to grow them in artificial media, the latter are prepared in such a way that they contain all the above ingredients. But even then, it is impossible to imitate the complexity of natural food of bacteria; hence they represent only poor substitutes for natural supply. In growing artificially, some species may require special ingredients for growth; as for example, hæmoglobin for Hæmophilic group of organisms; serum or white of an egg for Corynebacterium diphtheriæ; albuminous fluid, such as hydrocele, ascitic or ovarian fluid for Neisseria gonorrhœæ, Neisseria meningitidis, etc.; sugars (maltose) for fungus; glycerin for Mycobacterium tuberculosis hominis, and so on. When food supply fails, bacteria either degenerate or form spores and then ultimately die out. At this stage, transference to rich media will not be able to revive them. Again, during the process of growth, bacteria produce excretory products which may be unfavourable to their own vitality. These effete substances soon diffuse out into the medium and prevent growth.

Reaction of the medium in which an organism is allowed to grow is also an important factor. The majority of the bacteria prefers a medium with a slightly alkaline reaction of pH about 7·6. Small variations in the reactions, particularly to the side of acidity, may inhibit the growth altogether. Cholera vibrio, however, is the only organism which can grow in a highly alkaline medium, such as "Dieudonné's blood-alkali agar."

2. ***Gaseous environments:***—Bacteria vary in their capacity to grow with or without a supply of oxygen which is derived mainly from the atmosphere. Some are able to flourish in an atmosphere containing a free supply of oxygen, whereas others are unable to grow in its presence. According to their ability to grow in the presence or absence of free oxygen, bacteria are divided into two groups (a) aerobic and (b) anaerobic.

(a) **Aerobic:**—These require free oxygen for the maintenance of their vitality.

(i) *Obligatory aerobes:*—They can live and grow only when oxygen is freely supplied. The common examples are Pasteurella pestis, Hæmophilus influenzæ, Neisseria gonorrhœæ and Bacillus subtilis.

(*ii*) *Facultative aerobes:*—The organisms are truly anaerobes and therefore prefer to grow without oxygen, but in cases of necessity they can do so in its presence as well.

(b) **Anaerobic:**—Here the presence of oxygen is injurious to their growth and therefore they can flourish only when oxygen is excluded.

(*i*) *Obligatory anaerobes:*—These are unable to grow in the presence of free oxygen, *e.g.*, Clostridium group of organisms. They get their supply of oxygen indirectly by chemical decomposition of proteins and carbohydrates of the infected muscles.

(*ii*) *Facultative anaerobes:*—The organisms are truly aerobes and therefore prefer to grow in the presence of oxygen but are capable of living without it. Majority of the pathogenic bacteria are included in this group.

Apart from the above, there are certain other bacteria which prefer to grow best under conditions of lowered oxygen tension. These are called *micro-aerophilic*.

3. ***Presence of moisture:***—Some degree of moisture is essential for the continued growth of bacterial species. It is for this reason that all artificial culture media should contain sufficient fluid, specially at the bottom, as water of condensation. Different species of bacteria vary a great deal in their power to resist drying. Thus while cholera vibrio resists drying for 2 to 3 hours, Staphylococcus aureus will survive 10 days and Corynebacterium diphtheriæ still more. Again, spore-bearing bacteria withstand drying for a much longer period. Anthrax and tetanus spores can survive desiccation for years.

Note: In actual practice, it should always be seen that the culture media are moist and there is sufficient water of condensation at the bottom. Many Streptococci and sometimes, Diplococcus pneumoniæ, Neisseria gonorrhœæ and Neisseria meningitidis will not grow on a dry medium. Hence they may be missed and a wrong information reported.

4. ***Temperature:***—All the metabolic activities of bacteria are greatly influenced by the external temperature to which they are subjected. The range of temperature at which micro-organisms grow is subject to wide variations among different species, each one having an optimum and a minimum temperature. For every kind of bacterium there is a certain temperature at which it grows best and this is called the *optimum temperature*. There is also a maximum and a minimum temperature, above and below which, bacteria do not grow but may remain alive. For most of the pathogenic organisms, the optimum temperature is very near blood-heat, viz., 37°C. It should, however, be remembered that the optimum temperature depends upon the habitual environment in which the particular species is accustomed to exist, *i.e.*, the temperature of their natural habitat. In the laboratory, this constant optimum temperature is maintained by an incubator. In this country, during summer time, however, luxuriant growth takes place outside an incubator, *i.e.*, at room temperature. According to the temperature requirements for their growth, bacteria may be divided into two groups,

(a) mesophilic and (b) thermophilic. *Mesophilic* organisms are those which require an optimum temperature between 20°C. and 40°C. Majority of the pathogenic bacteria are included in this group. *Thermophilic* organisms are those which require an optimum temperature between 60°C. and 70°C. Such bacteria are not included under medical Bacteriology.

5. ***Effect of light:***—Direct sunlight is very injurious to bacterial growth. Similarly, green light and violet rays, such as, ultra-violet rays, X-rays and electric light are also injurious. All culture media after inoculation are placed in an incubator, the inside of which is dark. In this way, bacterial growth is protected from the action of direct sunlight. It is also for this bactericidal effect that direct sunlight is often employed as a natural disinfectant by many people in the tropics.

PRODUCTS OF BACTERIAL GROWTH: The bacteria, while growing in artificial media or in the animal body, produce various substances, the nature of which may vary according to the environmental conditions under which they are developed. Some of these substances may produce injurious action in the animal tissues and are referred to as bacterial poisons or toxins, while others are of the nature of enzymes or ferments which bring about important chemical changes in the surrounding medium. Their knowledge is being utilised in the biochemical study of bacteria and in the manufacture of various substances for industrial purposes. Again, in some cases, the bacteria may form pigments which give them their species characters. All the products of bacterial growth are considered below in detail.

1. ***Bacterial poisons:***—These are produced by the organisms during the process of their growth and are, in most cases, specific products of bacteria.

(a) **Exotoxin or extracellular toxin:** These specific bacterial products are supposed to be soluble secretions, passing readily from the bacterial cells into the liquid culture medium; hence they can be obtained from the medium after removal of the bacteria. Introduction of unglazed porcelain candles in the method of filtration of fluid medium in which bacteria are grown, considerably advanced the scientific study of extracellular toxins. In this way, the toxin contained in the fluid medium is obtained absolutely free from the organisms, thus facilitating the study of its action in a highly pure condition. Roux and Yersin (1888) first isolated diphtheria toxin by means of this kind of filtration; in a similar way tetanus toxin was discovered by Kitasato (1891).

The following is a list of organisms which produce true exotoxins:—

(i) Organisms of the Clostridium group (Cl. tetani, Cl. botulinum, Cl. welchii, etc.).

(ii) Corynebacterium diphtheriæ.

(iii) Eberthella dysenteriæ, Shiga.

(iv) Streptococcus scarlatinæ.

Note:—Recently an exotoxin has also been prepared from Staphylococcus.

It may be remarked here that all exotoxins cannot be regarded as active secretions of bacteria. Because it has been seen with some organisms, such as C. diphtheriæ and Cl. tetani, that maximum amount of toxin production results after some time of active growth and when the process of breaking down or autolysis of the bacteria begins to occur. Thus a definite statement cannot be made that all exotoxins are secretory products of bacteria. The peculiar property which characterises exotoxins is the readiness with which the latter can be separated in an active form from bacterial suspensions by filtration. The various other properties which serve to differentiate exotoxins from another kind of toxin (endotoxin), is shown later in a tabular form.

(b) **Endotoxin or intracellular toxin (Pfeiffer):**—In this case the poisons are not liberated in the liquid medium in which the bacteria grow but are linked more or less firmly to the bacterial body. Hence if a fluid culture, containing such organisms, is filtered through porcelain, the filtrate has little or no toxic effects, whereas the residue is very poisonous. Most of the common pathogenic bacteria produce endotoxins.

The main differences between the properties of exotoxin and endotoxin are shown in the following table.

Exotoxin	*Endotoxin*
1. Filterable.	1. Not filterable.
2. Usually fatal in very small doses.	2. Large amounts are required to produce fatal results.
3. Delicate and thermolabile.	3. Relatively thermostable.
4. Highly antigenic and capable of producing antitoxic serum.	4. Antigenic but not anti-toxinogenic.
5. They have specific effects on particular tissues of the body and their action can be assayed by pharmacological and other scientific methods.	5. No specific effect on any particular tissue and their actions cannot be recognised like those of the exotoxins.

(c) **Aggressins:**—These are substances which are produced only in the animal body by bacteria and are definitely related to their virulence (*Bail's theory*). Their action is exactly opposite to that of *opsonins*, and they enhance the bacterial activities by preventing the leucocytes to come near them and thus escape their destructive action. Their action is supposed to be the production of a negative chemiotactic phenomenon. The organisms of gas-gangrene are one of the best examples which produce aggressins.

Apart from the above poisonous products elaborated by the bacteria, the cytoplasm of the bacterial bodies is, in many cases, injurious to animal tissues. This protein residue which remains after removal of all exo and endo, toxins, may give rise to localised inflammatory lesions when injected into animals. This bacterial protein is the same in all species and has no specificity. Its action is similar to that of "*Non-specific Protein Therapy,*" exerting a general stimulus to the antibody-forming elements of the body viz., the reticulo-endothelial system.

2. ***Ferments:***—These are special kinds of proteins acting as catalytic agents. They take part in the reaction but are not used up in the process. Like toxins, they are both extra and intra, cellular The following are some of the common bacterial ferments:

(a) **Proteolytic ferment:**—This is responsible for the decomposition of dead animal and vegetable matter in nature. In the laboratory, common examples of proteolytic action may be seen in the liquefaction of gelatin, fibrin and coagulated blood serum. Thus, it will be seen that if properly sterilised to prevent the growth of bacteria, substances like meat, fish and albuminous materials will keep for a long time. This knowledge is applied in the artificial preservation of meat, fish and other foodstuffs, for commercial purposes.

(b) **Coagulase:**—This is a milk-curdling ferment, often demonstrated during the study of the biochemical properties of many pathogenic organisms. This ferment action is sometimes used as an ancillary method in the diagnosis and identification of bacterial species. Many organisms of the colon group produce marked coagulation of milk due to their production of this ferment.

(c) **Amylase:**—This is a ferment, splitting carbohydrates, specially starch. Although this reaction is not much used in the study of bacteria, it is extensively employed in the preparation of acetone for commercial purposes.

(d) **Lactic acid fermentation:**—It is produced by the activity of certain bacteria in media containing carbohydrates and albuminous substances. Aerobacter ærogenes (B. lactici ærogenes), organisms of the Lactobacillus group and some other varieties of the colon group of bacteria can decompose many di and mono, saccharides, producing lactic acid as one of the products. It is this lactic acid which changes the reaction of the culture media in which the bacteria grow. The common souring of milk, used both for household and commercial purposes, depends on this biochemical process.

(e) **Oxydase (oxidising ferments):** The commonest example is Bacterium aceti which produces acetic acid from a weak solution of alcohol. It was this ferment-action which was first studied by Louis Pasteur and which converted him from a chemist to a bacteriologist. The ethyl alcohol is oxidised with the formation of acetic acid according to the following formula:

$$\underset{\text{Ethyl alcohol}}{C_2H_5OH} + O_2 \quad \underset{\text{Acetic acid}}{CH_4COOH} + H_2O.$$

Although we do not employ this ferment activity in the common laboratory study of bacteria, this knowledge is utilised in the commercial manufacture of vinegar.

(f) **Alcoholic fermentation (Zymase):**—The most important organism which produces this ferment is the yeast which converts the monosaccharide, dextrose, into alcohol and carbon dioxide, as shown in the following formula:—

$$\underset{\text{Dextrose}}{C_6H_{12}O_6} + \text{Zymase} \quad \underset{\text{Ethyl alcohol}}{2C_2H_5OH} + 2CO_2$$

This knowledge is applied in the industrial production of wine and beer.

3. ***Chromogenic products:***—Many bacterial species have the power to produce pigments, e.g.:—

Staphylococcus aureus—golden yellow pigment.

Pseudomonas aeruginosa (B. pyocyaneus)—blue pigment.

Serratia marcescens (B. prodigiosus)—red pigment.

Chromobacterium violaceum (B. violaceus)—violet pigment.

The pigments produced by different bacteria differ in colour and in their solubility. Bacillus pyocyaneus produces a water soluble pigment which diffuses through the medium; whereas in the case of Staphylococcus aureus, the pigment does not diffuse out in the medium but remains within the bacterial cells and only the colonies become coloured. The presence of oxygen is necessary for the development of chromogenic properties. When grown anaerobically, this chromogenic power is lost and the same result may follow heating to 55 C. and prolonged culture on artificial media.

Significance:—The pigment-production by bacteria does not serve any physiological purpose but is only concerned in the differentiation of bacterial species or varieties.

CHAPTER IV

STERLISATION

It is essential that no extraneous organisms should get access to the culture media which are used for the cultivation and study of the characters of an individual bacterium. To ensure this, it is necessary to obtain the organism in *pure culture*, which is the first requisite of a bacteriological research. All apparatus and culture media must therefore be free from extraneous organisms before use and the process by which this is carried out is called ***sterilisation.***

METHODS:—There are various methods of sterilisation depending on the nature and composition of the articles to be sterilised and hence one method cannot be employed for all purposes. Each one has its own particular use with well-defined limitations. The following are some of the common methods:—

1. *Sunlight (Ultra-violet rays).*
2. *Drying.*
3. *Heat.*
4. *Sterilisation at lower temperature.*
5. *Sterilisation by filtration.*
6. *Sterilisation by chemicals.*

The usual methods of sterilisation are some form of heat, either dry or moist; the latter being the more effective. High temperature (100°C. or over) or low temperature (below 100°C.) may be used according to the stability of the substances it is desired to sterilise.

Sterilisation can be effected by means of antiseptics but they are not usually employed in the preparation of culture media as the presence of the chemical which destroys the contaminating organisms, will either kill or prevent the growth of the bacterium under study.

SUNLIGHT:—The ultra-violet portion of the sunlight is active and lethal, not only to bacteria, but to other unicellular organisms like the amœba, as well as, to the living cells of animal and plant tissues. The mode of action of the ultra-violet light is essentially a chemical one, depending on the presence of free oxygen and producing an alteration in the surface layer of the cells. Bacteria suspended in water are readily destroyed by exposure to sunlight. This is one of the natural methods of sterilisation in cases of water in tanks, rivers, lakes, etc. The strong current is in addition a factor in the sterilisation of running waters. This explains why water from springs and rivers, with strong currents, is wholesome, whereas stagnant waters are not so.

In our country, the powerful sunlight is a great disinfecting agent. Partly due to its actinic activity and partly to heat, the rays of the sun act as a natural destructive agent to many pathogenic organisms. It has been definitely found by Rogers and others that clothes, moistened with the fæces of cholera and typhoid cases, are rapidly sterilised on exposure to direct sunlight. Thus, although the tropical sun is associated with an uncomfortably hot climate, yet it is a boon to the people for its powerful germicidal effects. In a country like India, the sterilising effect of the tropical sun is certainly beneficial and counteracts the evils, to a greater or less extent, of the careless dissemination of the infecting agents through fæces, sputum and other discharges by people of the illiterate class, who are ignorant about the common principles of hygiene. Besides, the people of the poorer section employ it as a natural mode of disinfectant by exposing their soiled clothings to the sunlight.

DRYING:—As already mentioned, all bacteria, in their vegetative state, require some amount of moisture for their continued growth; hence, drying acts as a sterilising agent and comprises another natural mode of destruction of bacteria. This, however, does not apply to spore-bearing organisms which can resist drying very considerably.

HEAT:—This is one of the most powerful and efficient methods of sterilisation. It acts by coagulating bacterial protoplasm and thereby killing them. Heat may be applied in two forms, dry heat and moist heat. There is a marked difference between the action of the two forms of heat. Moist heat is, by far, the more powerful agent because of its greater and quicker power of penetration. When any bacterium is exposed to the action of *dry heat*, such as a current of hot air, it is first dehydrated before its protoplasm coagulates. Such a process naturally takes some time. Of course, when the temperature is raised very much, it is burnt to death. The action of *moist heat*, on the other hand, is entirely different. As soon as steam comes in contact with any object it passes from the gaseous into the liquid state, liberating at the same time, a large amount of heat which is absorbed by the object. The latter is thus quickly heated. Further heating of the object is then effected by conduction. Hence the rapid heating coagulates the bacterial protoplasm and kills the organism.

A. ***Dry heat:***—This is applied in the following forms, such as red heat, flaming and a current of hot air (Hot-air steriliser).

(a) **Red heat:**—It is used for platinum wires, needles, forceps, searing spatula, etc.

(b) **Flaming:**—Scalpels, needles, the mouths of culture tubes, cotton-wool plugs, glass slides and coverslips are sterilised by passing the articles through the flame of a Bunsen burner without allowing it to become red hot. This method is also used to sterilise basins and other materials which are moistened with rectified spirit and then lighted.

(c) **Hot-air oven (Steriliser)** (Fig. 29):—This was first introduced by Pasteur in 1876. This is used for sterilisation of all glass materials, such as Petri dishes, test tubes, flasks, pipettes, throat swabs, etc., before being used for bacteriological purposes. These articles should be wrapped in paper prior to being placed in the oven. When removed, the paper will appear slightly charred, if the oven has been working properly. The desired temperature that is to be attained is 150° to 200°C. and exposure to this temperature for an hour is sufficient to effect sterilisation.

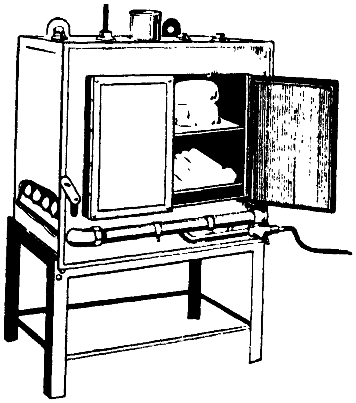


Fig. 29.
Hot-air steriliser.

Description of the apparatus (Fig. 30):—This is a rectangular box of sheet iron or copper with double walls, furnished with a door and supported on legs. The chamber consists of an outer and an inner case, having an air-space of nearly an inch between them. The outer wall of the apparatus is provided with a thick asbestos sheet which lines all the surfaces excepting the bottom. The apparatus is heated by a ring of gas burners which are arranged at the bottom on all sides of the chamber excepting the front. The hot air passes between the two jackets from below upwards, plays round all the sides of the inner case and escapes through the holes on the top of the outer case, which serve the purpose of regulating the current of air. The top of the apparatus is perforated with a couple of holes, one for the thermometer registering up to 250°C., which is inserted through a cork, and the other for a thermo-regulator if necessary. The thermometer passes down the interior of the apparatus so that its bulb should reach about the top of the inner chamber. Two or more perforated movable shelves are provided inside the chamber for putting in the articles for sterilisation. Electrically heated ovens are now often used in which the temperature can be maintained at the desired height by means of a regulator.

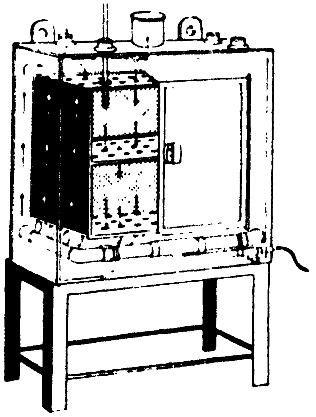


Fig. 30.
Sectional diagram of hot air steriliser.

Points to be remembered in using the apparatus:—

(i) The glass vessels should be perfectly dry. If any water remains in the glass ware, the latter is liable to be cracked.

(ii) The oven must be cold before the apparatus is worked. All the materials are first put in the chamber, which is then heated to 150°C. From this point, the apparatus is worked for one hour during which period, the temperature may rise up to 200°C. The gas is then turned off and the oven is allowed to cool. The articles are kept in it, after sterilisation, till the temperature falls below 100°C., as sudden cooling is liable to crack the glass materials.

B. Moist Heat:—This is commonly employed as ordinary boiling or steam.

(a) **Boiling:**—This is useful for the sterilisation of syringes, instruments, gloves, rubber goods, etc. Boiling applied for twenty minutes to half an hour is sufficient to kill all the organisms including the spore-bearers. The addition of a 1 per cent. solution of sodium carbonate to the boiling water hastens the destruction of spores and prevents the rusting of metal objects sterilised in this way. Addition of 2 to 5 per cent. phenol to boiling water destroys the anthrax spores in 10 to 15 minutes.

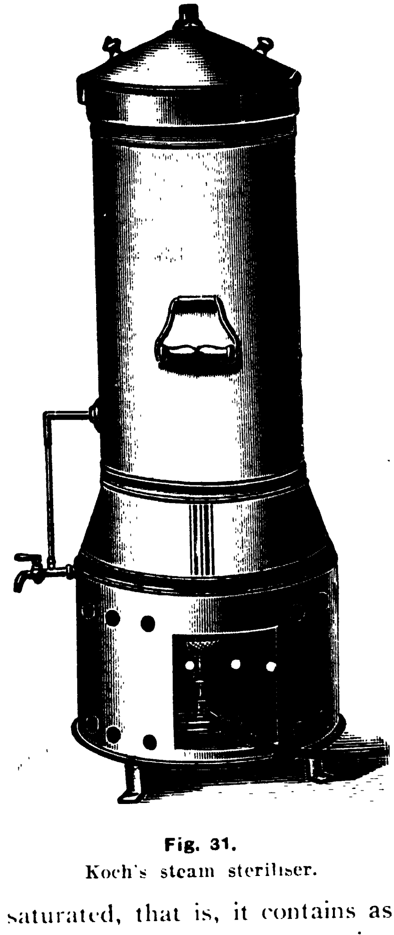


Fig. 31.
Koch's steam steriliser.

(b) **Steam:**—This may be used at a more or less constant temperature of 100 C. when it is without pressure (live or flowing steam) or at temperatures far exceeding 100 C. when it is used under pressure (compressed steam). The former is applied in a *Steam steriliser* and the latter in an *Autoclave*. The efficiency of compressed steam is much greater than that of live steam. In both the cases, the steam is saturated, that is, it contains as much water vapour as its temperature permits. When heat is applied to steam, its temperature rises gradually and finally it becomes dry. Such a steam is said to be superheated or unsaturated and its disinfecting power is much less than that of saturated steam.

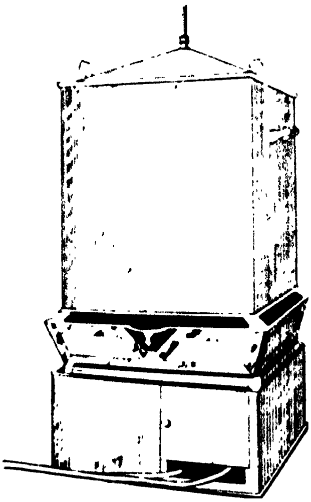


Fig. 32.
Arnold's steam steriliser.

(i) *STEAM STERILISER (STEAM AT 100°C. WITHOUT PRESSURE):*—This is employed for the sterilisation of media which are decomposed at higher temperature of the autoclave, *e.g.*, gelatin and carbohydrate media. The method is extensively used in bacteriology, and the apparatus most commonly employed is "Koch's Fig. 31) or Arnold's steam steriliser" (Fig. 32).

DESCRIPTION OF THE APPARATUS (Fig. 33):—The apparatus consists of a cylindrical or rectangular vessel of galvanised iron or copper, supported on legs, and covered externally by some bad conductor of heat, such as felt or asbestos. It is provided with a movable lid which has a hole for the thermometer. Inside, there is a perforated tin diaphragm supported four inches above the bottom and a tap is fitted below for the withdrawal of water. The flask or other objects to be sterilised are placed on the diaphragm. Water is placed below the diaphragm to the depth of 3 inches and is boiled by means of a ring burner. Steam at 100°C. eventually ensues, fills the space above the diaphragm and surrounds any flask or other objects standing on it. The advantages are that both the container and the contents are sterilised and as the medium is surrounded during sterilisation by an atmosphere of steam, no evaporation can take place from them. It is advisable to cover the cotton-wool plug of tubes or flasks with paper to prevent its becoming drenched with condensed steam.

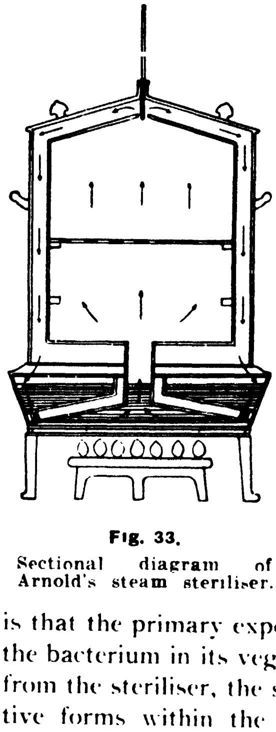


Fig. 33.
Sectional diagram of Arnold's steam steriliser.

STERILISATION IS EFFECTED IN TWO WAYS:—

(1) *One exposure for one and a half hours.* Media such as nutrient broth or agar are usually sterilised by this method. On the other hand, media containing gelatin and sugars cannot be sterilised in the above way, as such a lengthy exposure is detrimental. The method which is employed for the latter is known as "Intermittent or fractional sterilisation."

(2) *Exposure at 100°C. for 20 minutes each day for 3 to 5 successive days (Intermittent or fractional sterilisation):*—The principle of this method is that the primary exposure (sterilisation on the first day) is sufficient to kill the bacterium in its vegetative stage, the spores remaining alive. On removal from the steriliser, the spores, being in a favourable medium, assume vegetative forms within the next twenty-four hours which intervene before the second heating. The vegetative forms are killed by subsequent heating. In case any spores have not developed into vegetative forms during the first twenty-four hours, the process is repeated on the third day. The third heating is thus an additional precaution. The process may be repeated if necessary.

(ii) *AUTOCLAVE (STEAM UNDER PRESSURE):* This is the commonest and the most efficient means of sterilisation and is effected in the autoclave, first introduced by Pasteur in 1876. This method cannot be employed for the sterilisation of sugar and gelatin media, as the former is decomposed at such a high temperature and the latter cannot be solidified on cooling.

DESCRIPTION OF THE AUTOCLAVE (Figs. 34 & 35):—The autoclave consists of a cylinder of gun-metal which is supported in a cylindrical sheet-iron case. It is provided with a removable lid which is fastened down to the boiler with screws and nuts and is rendered air-tight by means of an asbestos washer. The lid is furnished with (1) a stopcock, to relieve the pressure; (2) a gauge, for indicating the pressure and temperature and (3) a safety-valve, which can be set to "blow off" at any desired temperature. The contents of the apparatus are more or less the same as in the "steam steriliser," *i.e.*, a collection of water to a depth of about 3 to 4 inches at the bottom with a perforated diaphragm supported above the level of the water. The apparatus is heated from below by a ring of gas burners.

PRINCIPLE OF THE AUTOCLAVE (STEAM AT HIGH PRESSURE):—For boiling, the vapour pressure of a liquid must be equal to the pressure of its surrounding atmosphere. As the autoclave is a closed vessel, the water may be caused to boil at a temperature higher than its boiling point (100°C.) by increasing the pressure inside it. Thus the temperature of the steam inside the autoclave can be raised by regulating the pressure inside. The temperature employed is usually 115°C or 120°C. A pressure of 15 lbs. to the square inch in addition to the ordinary atmospheric pressure is necessary to raise the boiling point of the water to 120°C. When bacteria, including the spore-bearers, are exposed to such a temperature for 20 to 30 minutes, they are all killed. In such an apparatus the desired temperature is maintained by a safety-valve which is so adjusted as to blow off at the requisite pressure.

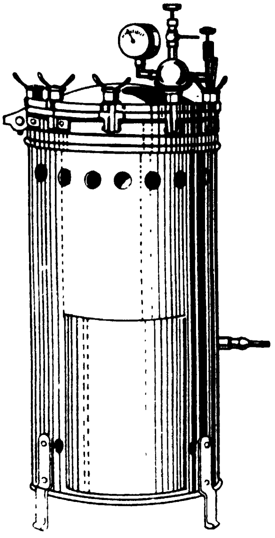


Fig. 34.
Autoclave.

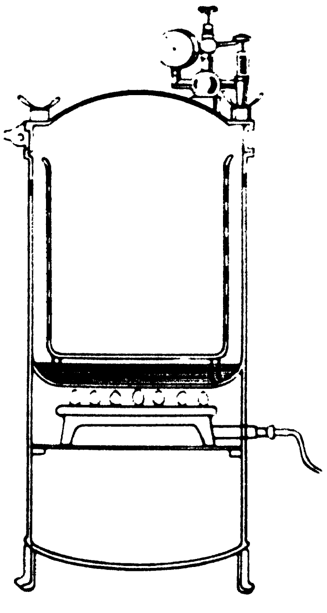


Fig. 35.
Sectional diagram of autoclave.

DIRECTIONS FOR USING THE AUTOCLAVE:—Sufficient amount of water must remain at the bottom of the cylinder, and its depth should not be less than 3 to 4 inches. Put the perforated diaphragm inside the cylinder and arrange on it the materials to be sterilised. Place the lid in position and apply heat. Adjust the safety-valve to the required pressure and leave the stopcock open. As the autoclave is heated, air is forced out of the tap and eventually steam ensues. Make sure that all the air has been expelled from the cylinder which is evidenced by steam issuing freely. Then turn off the tap. The pressure now rises until it reaches the desired level, when the safety-valve opens and the excess of steam escapes. From this point reckon the requisite time for sterilisation which is 20 to 30 minutes as the case may be. When sterilisation is complete, put out the burners and allow the autoclave to cool well below 100°C. Now open the stopcock to let out the excess of steam. Remove the lid and take the materials out.

PRECAUTIONS:—

(i) See that sufficient water is present at the bottom so that there is a residuum when steam is fully up, otherwise the latter is superheated and the pressure gauge will not indicate the correct temperature.

(ii) See that all air from inside the apparatus is driven out, otherwise a mixture of air and steam being present, the reading in the pressure gauge would be misleading.

(iii) Allow the apparatus to cool below 100°C.; otherwise with sudden release of pressure, the liquid media, being still at a temperature of above 100°C., are exposed to ordinary atmospheric pressure and thus will boil violently and are liable to be blown out of the flasks.

STERILISATION AT LOW TEMPERATURE:—

This method is used for sterilising (A) vaccines and (B) blood serum or media containing protein.

A. Vaccines:—These are sterilised by heating at 60°C. for one hour in a water bath (Fig. 36). Overheating should be avoided as the immunising power is damaged thereby.

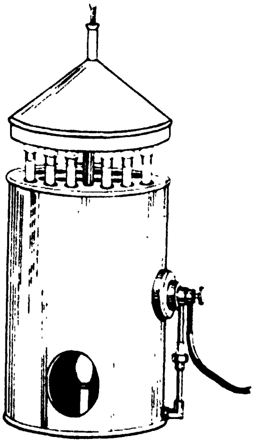


Fig. 36.
Vaccine bath.

B. Blood serum or other media containing protein:—A prolonged exposure to a temperature of 55° to 60°C. will kill many organisms in the vegetative forms but will not coagulate the protein. The media containing serum are sterilised as follows:—

(i) **Sterilisation without coagulation:**—This principle is utilised in the sterilisation of serum which may then be used for the preparation of serum media. Usually an exposure to this temperature in the water bath for one hour for 3 to 5 successive days is sufficient.

(ii) **Sterilisation with coagulation:**—This is done in a serum inspissator and the process is called inspissation. The requisite quantity of serum is put in a sterile test tube which is then heated in the inspissator for one hour at 75° to 80°C. for 3 to 5 successive days. This stiffens the serum without actually coagulating it as this temperature is just below the coagulation point of the serum.

SERUM INSPISSATOR (Fig. 37):—The apparatus consists of a rectangular chamber made of copper with a water jacket around it and is heated by gas from below. The temperature is controlled by a gas regulator. Inside the chamber there are special racks over which the tubed egg or serum media are placed in a slanting position. The racks can be adjusted by means of screws so as to place the tubes at the correct angles to attain the desired slopes. The tubes are kept in the chamber each day for one hour at 75° to 80°C. for 3 to 5 successive days.

STERILISATION BY FILTRATION:—This method is particularly used for (a) obtaining soluble toxic products (exotoxins) of bacteria from fluid cultures and also for the manufacture of exotoxins and (b) investigating "ultra-microscopic or filter-passing viruses." The instruments used for the purpose are called filter candles, the pores of which are too small to allow the bacteria to pass through. Filter surfaces are of three main varieties and are made of the following:—

(i) *Diatomaceous earth (Kieselguhr) filters:* The type filters are Berkefeld (German) and Mandler (American).

(ii) *Unglazed porcelain made from Kaolin and certain admixture of sand:* The type filters are Pasteur-Chamberland (French), Doulton (English) and Massen (German).

These two types of filters are made in the form of cylindrical hollow candles.

(iii) *Asbestos discs:* The type filter is Seitz. This form has the advantage that after use the discs are discarded and a new one is employed for each filtration.

Further details on filtration are described in the chapter on filterable viruses.

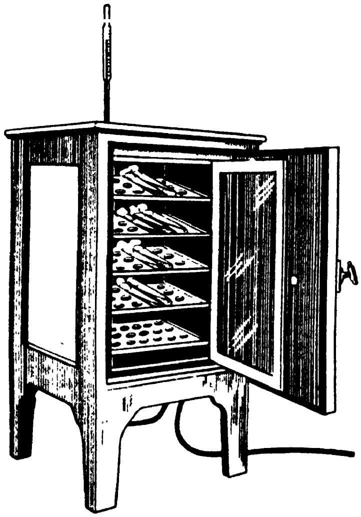


Fig. 37.
Serum inspissator.

STERILISATION BY CHEMICALS:—

1. ***Volatile antiseptics,*** e.g., chloroform. This is used in the proportion of 0·25 per cent. for sterilisation of serum for culture media. The chloroform can be removed by heating to 57°C. before the serum is used. It is also employed for preservation of serum and media in bulk.

2. ***Antiseptics of the phenol group:***—Phenol (carbolic acid) in 0·5 per cent. solution is used for preserving sera and vaccines. Carbol-saline (normal saline containing 0·5 per cent. phenol) is used as the vehicle for bacterial vaccines; the phenol inhibiting the growth of contaminating moulds. Lysol (2 per cent.) and cresol (5 per cent.) are chiefly used in the laboratory for sterilising surgical instruments, discarded cultures, infective materials such as sputum, and for disinfecting the hands and working tables.

3. ***Metallic salts:*** The most important in this group is a 1 in 1,000 solution of perchloride of mercury, particularly used for washing hands.

MAINTENANCE OF STERILITY

It is necessary that media, apparatus or other materials are to be kept in a sterile condition. To avoid subsequent contamination with aerial bacteria, non-absorbent cotton wool is frequently used in the bacteriological work. Cotton wool plugs or stoppers should be about 1½ in. long, 1 in. being inserted into the mouth of the tube and ½ in. projecting. They should fit firmly, but not so tightly as to render their removal difficult.

Tubes and flasks are protected from aerial contamination of dust and micro-organisms by means of cotton-wool stoppers. These may however be covered with sterile paper tied over them or sterile rubber stoppers may be used in some cases, particularly when stored for a longer period to avoid drying of the medium. *Petri dishes and pipettes* should be wrapped in papers before sterilisation and are to be taken out only at the time of use. *Capillary pipettes* are best sterilised with their ends sealed in copper boxes. *Ampoules* are sterilised with the neck sealed. *Measuring pipettes*, used for complement fixation test, are sterilised by wrapping them up in paper, the mouth end being closed with a pellet of cotton wool. *Syringes* may be kept sterile, by putting them in suitable test tubes, needles downwards. The syringe is fitted in the test tube with sterile cotton wool plug, the whole being covered with paper. This is arranged as shown in Fig. 38. The whole arrangement may then be sterilised in the autoclave and kept in stock, to be used whenever it is required.

Fig. 38.

Syringe sterilised and kept ready for drawing blood.

CHAPTER V

PREPARATION OF CULTURE MEDIA

The materials used for the preparation of culture media depend largely on the natural requirements of the particular organism. Most of the pathogenic bacteria with which we are concerned, require a medium which roughly corresponds to the composition and reaction of the body fluids and are derived from the flesh and fluids of animal bodies. In the early days, a watery extract of meat was considered to be rich in nutritive elements necessary for bacterial growth but it was soon found out that except the extractives and the salts, it did not contain much nourishment. Later on, to increase its nutritive value, it was necessary to add various split products of protein digestion, such as peptones. Although the quality of culture medium was in this way considerably improved, the only medium available was a liquid one which did not allow the isolation of bacteria in pure culture. It was Robert Koch who, in 1881, first introduced solid culture media in the study of bacterial growths, particularly for their separation in pure culture from a mixture. He added gelatin to his liquid medium and thus obtained the first solid culture medium. Such a medium, however, melts at 24 C., i.e., at a temperature much below that of the body and thus is unsuitable for the growth of many delicate bacteria which grow best at blood heat (37·5 C.). Agar (obtained from Chinese sea-weeds) which remained solid at a temperature much above that of the body and which does not melt below 98 C., was substituted for gelatin. Thus the basal media which are stocked in the laboratory are either a fluid medium, bouillon (usually called broth) or solid media, nutrient agar and gelatin (broth solidified by the addition of agar or gelatin). The great majority of bacteriological culture media are prepared from these basal media and enriching materials may be added for special purposes.

GENERAL TECHNIQUE

Preparation of glass ware:—

(1) All new glass wares should be cleaned with a 1 per cent. nitric acid to dissolve any excess of alkalis and subsequently treating them with a weak solution of caustic soda (one per cent.) to neutralise all traces of the acid. They may now be washed and cleaned in running water to remove any trace of the chemicals used.

Old glass wares, which have already been used for cultural purposes and which might contain actual growth of virulent micro-organisms, require a thorough and careful sterilisation before mechanical cleaning and washing. This may be done either by heating them in an autoclave or by prolonged boiling. After this process, they may be mechanically cleaned with fuller's earth or soap suds and washed thoroughly in tap water.

(2) After washing, all glass wares should be allowed to dry completely. This stage may be considerably expedited by keeping all materials in a suitable position to allow the water to run down by gravity. An equipment, like the one shown in Figure 39, is convenient for flasks, test tubes, etc. When they are thoroughly dry, the test tubes, flasks, etc., should be plugged with non-absorbent cotton wool. The Petri dishes, when dry, should be wrapped in paper.

(3) After plugging, they are sterilised in hot-air oven for one hour at 150° to 200°C. All care should be taken so that the temperature does not go above 200°C., because in that case a browning of the cotton plugs and charring of the paper occur, and the glass is apt to be blackened by the burning of the grease and other organic materials derived from the cotton plugs.

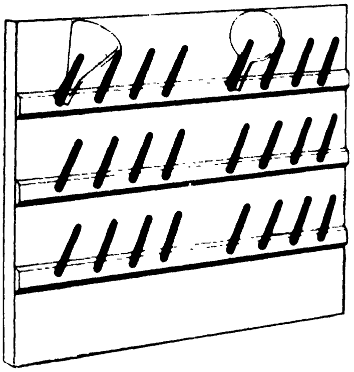


Fig. 39.
Draining board.

Recently the use of *screw-capped sterilised bottles* (Fig. 40) as supplied by the United Glass Bottle Manufacturing Co., London, has been advocated by McCartney in the preparation and storage of culture media (The Lancet, August 19, 1933). The bottles may be used repeatedly, after properly cleaning them and using fresh screw caps every time. As substitute for flasks and test tubes with cotton-wool plugs, they are very useful for small laboratories and long distance transit, as the media undergo very little desiccation during storage or transit and remain sterile indefinitely.

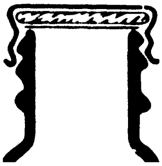


Fig. 40.
Section of a screw-capped bottle in the preparation and storage of culture media. The shaded area shows the position of the medium for a slope culture. (By kind permission of "the Lancet").

Methods of clearing media:—

(a) **By adding white of eggs:** —Clearing of media is carried out with eggs if the media do not contain any coagulable protein. The white of some eggs (one egg to each litre of medium) is beaten up with a little water and added to the medium, which, if hot, as in the case of the melted agar or gelatin, must first be cooled to 50° to 55°C. The flask containing the medium is heated, shaken vigorously to break up the coagulum and again steamed for 15 minutes. The medium is now filtered. The coagulation of the albumin precipitates the fine particles present in suspension.

(b) **By filtering through cotton wool:** — Filtration is best done through cotton. A square piece of absorbent cotton is split horizontally giving two squares of equal size and one is placed above the other at the bottom of a large glass funnel, in such a way that the direction of the fibres of the one is at right angles to that of the other.

The funnel should be warmed in the case of gelatin and agar. The coagulated albumins settle on the cotton and act as a part of the filter.

(c) **Filtering through paper:**—Many media may be efficiently cleared by filtration through close filter paper without the aid of coagula.

Setting of media:—The larger the surface area available for stroke culture, the more useful is the medium for the isolation of separate colonies from a mixture. Solid media in tubes are usually set either in an *upright* or *sloped position*. The upright tubes are used for *stab* or *shake cultures* or for storing a medium in small lots (e.g., making of plates). A slope is designed to give a wide surface area. Upright tubes are simply allowed to stand vertically to solidify. For slopes, the tubes are allowed to solidify on an incline. For gelatin tubes it may be necessary to slope them in a tray with fairly cold water. Plates will set fairly quickly when left horizontally on a table.

PREPARATION OF BASAL MEDIA

NUTRIENT BROTH (BOUILLON)

Substances required:—

Fresh heart muscle (minced), preferably from beef	...	500 gms.
Water	...	1,000 c.c.
Peptone (Witte's)	...	10 gms.
Sodium chloride (chemically pure)	...	5 gms

Technique:—

(i) Making of watery extract.
(ii) Preparation of bouillon.
(iii) Adjustment of reaction (Titration).
(iv) Filtration.
(v) Tubing of media.
(vi) Sterilisation.

(i) **Making of watery extract of meat:**—Fresh heart muscle (preferably ox-heart) is used for the purpose. It is freed from fat and blood vessels and minced in the mincing machine thrice to make it very fine. 500 gms. of minced meat are added to 1 litre of tap water in a clean glass jar and covered. The jar is kept in the ice chest over-night. Next morning, it is taken out and boiled gently in a metal vessel for half an hour. It is then filtered through ordinary filter paper and the filtrate is made up to the original volume with distilled water. The final product is clear and light yellow in colour and possesses a sweetish odour.

(ii) **Preparation of bouillon:**—Such a meat extract is not a suitable culture medium as the proteins are removed by coagulation and filtration and the salt content is also poor. To make good the deficiency in the above, two ingredients, viz., peptone (Witte's) 10 grams and chemically pure sodium chloride 5 grams, are added. The mixture is [illegible] steamed in a steam steriliser for 20 minutes to dissolve the peptone.

(iii) **Adjustment of the reaction:**—Correct adjustment of the reaction of culture media is essential for their proper utility. Bacteria are highly sensitive to differences in the acidity and alkalinity in reaction, i.e., to differences of hydrogen-ion concentration. Excessive variations may completely inhibit their growth while moderate variations may markedly modify the cultural characteristics. Majority of the pathogenic microbes prefer a slightly alkaline medium, e.g., a reaction similar to that of body fluids. The following are the various methods by which the reaction of broth is standardised:—

(a) *SIMPLE METHOD:*—The simplest method consists in adding acid or alkali as the case may be till the medium is slightly, but distinctly, alkaline to litmus paper. The test paper must be immersed in the liquid, and the blue paper should remain unaffected.

(b) *PHENOLPHTHALEIN METHOD:*—(Eyre's method)—The titration of culture media with phenolphthalein as an indicator is not accurate, as it takes no account of the "Buffer" substances present in considerable quantities in culture media (e.g., peptones, proteins, phosphates, etc.). Hence it is losing favour and is rapidly being replaced by hydrogen-ion concentration methods. According to Eyre's scale, the reaction or titre of media is expressed by the number of cubic centimetre of N/1 alkali or acid required to render one litre of the medium exactly neutral to Phenolphthalein. The sign + (plus) is placed before the number, if the original solution reacts to acid and the sign − (minus), if it is alkaline. For example, when the reaction is expressed as + 10, it means 10 c.c. of N/1 NaOH are required to make a litre neutral

(c) *PH (HYDROGEN-ION) METHOD:*—This is the most accurate method. PH values can be determined electrically, but colorimetric methods of estimating pH values are generally used in Bacteriology. The indicators used consist of organic colouring matters and each has a definite pH range and is only of use within that range. The neutral points do not coincide and what is acid by one indicator may be alkaline according to another. For instance, litmus is red at pH 5 or less, purple at pH 7 and blue at pH 8 or over. Phenol red solution is yellow at pH 6·8, red at pH 8·0 and pink at pH 7·4. Several firms sell indicators of varying pH range. Standard tubes of phenol red of known pH values with definite concentration are very suitable for media work. The hydrogen-ion concentration is thus standardised by the Comparator method, either using standard colour tubes and a Comparator box or Hellige Comparator with non-fading standard colour glass plates.

USE OF STANDARD COLOUR TUBES:—The following materials are required for the purpose:—

1. A set of 8 sealed standard tubes having a range of pH varying from 6·6 to 8·0 with a difference of pH 0·2 between each tube and the next. (Fig. 41).

2. Cordite (hard white glass) tubes of a uniform thickness of wall and bore and identical with standard pH tubes.

3. A colour-comparator box, consisting of a rack to hold two rows of three cordite tubes and having a ground glass at the back to disperse the light. The use of the comparator obviates the interference of colour judgment due to the colour of the media.

4. Phenol red solution (0·01 per cent. in distilled water).

5. Micro-burette measuring upto 0·01 c.c.; pipettes of 5 c.c. and 0·5 c.c.; N/10, N/1, and 10N, NaOH.

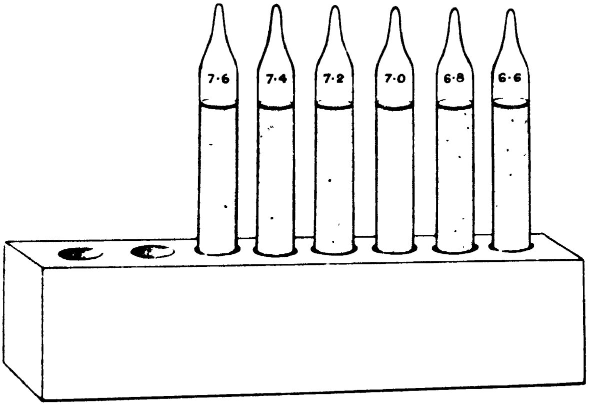


Fig. 41.
Colour tubes of standard pH values.

Method:—In the comparator rack the tubes are arranged as follows (Fig. 42). In 1 and 3, standard pH tubes are placed and if a reaction of pH 8 is required, standard tubes of pH 7·8 and 8 respectively, are placed in position. In front of 1 and 3, i.e., in 4 and 6, tubes containing medium only are placed. In 2, a tube containing distilled water only is placed. In 5, a tube containing 5 c.c. of the medium and 0·5 c.c. of a 0·01 per cent. phenol red solution is placed. It will be seen from the above that the light transmitted through each pair of tubes passes through (i) a tube of the medium, and (ii) a solution of an indicator. The alkali is now added in tube 5 in the form of N/10 NaOH and is run out in measured quantity from the microburette. The amount of alkali necessary to bring the tint to match with the tube 3 is noted. The process is repeated and the average of two readings is taken. From the amount of alkali used for 5 c.c. of broth, it is easy to calculate the amount of decinormal, normal or decanormal NaOH to be used per litre to give it a pH of 8.

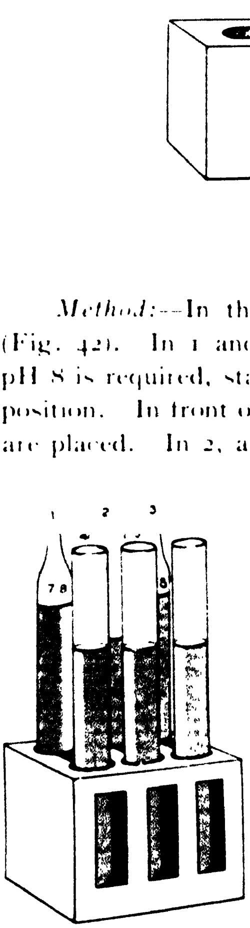


Fig. 42.
Comparator box set up for the adjustment of reaction of the medium.

The defect of this apparatus is that the colour of the standard tube gets fainter and fainter, specially on frequent exposure to light. Hence Hellige comparator with permanent non-fading standard colour glass plates is recently gaining favour.

HELLIGE COMPARATOR:—The instrument consists of the following (Fig. 43):—

(i) A metal housing.

(ii) One prism for bringing the colour fields into juxtaposition and consequently increased accuracy of reading of the registrations.

(iii) Two acid-proof vitrified troughs with plane-parallel walls to receive the liquid to be analysed as well as the liquid with the addition of indicator.

(iv) Four gauged test tubes for measuring the test solution.

(v) A colour disc with a series of round standard colour plates (9 or 10) disposed in a revolving circle, each calibrated for the particular indicator and representing definitely determined pH values with a progressive interval of 0·2 pH. A special colour disc being used for each range of indicators.

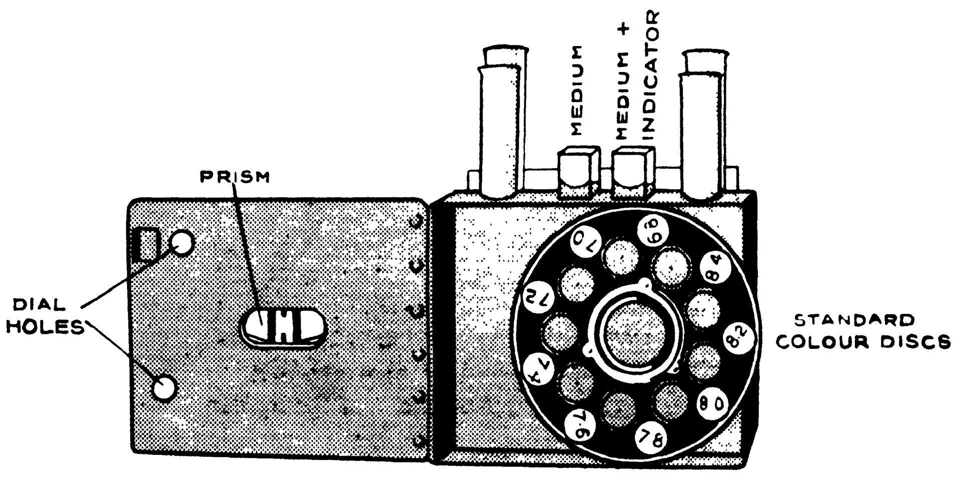


Fig. 43.
Hellige comparator.

(vi) A white or blue frosted glass plate.

(vii) 100 c.c. bottle of indicator solution and a measuring pipette.

(viii) A stand to fix the comparator at eye-level on the working table.

Method:—(Fig. 44). A measured quantity (10 c.c.) of filtered sample is placed in each of the two troughs. To one in the right hand receptacle is added the indicator solution (0·5 c.c. of 0·02 per cent. phenol red), as specified in the disc. Now if a reaction of pH 8 is required, by revolving the disc, the corresponding glass standard is placed opposite the tube containing the sample without indicator; the number is read off from one of the dial holes. N/20 NaOH is then added to the sample containing the indicator and the colour is matched through the aperture of the metal case. Turbidity or colour of samples being compensated by interposing a tube containing the sample alone between the glass standard and the light against which the fields are compared. The amount of alkali required to titrate 1,000 c.c. of the sample to obtain a reaction of pH 8 can easily be calculated out.

It should be remembered that with each steaming and autoclaving, pH value falls by 0·2, and this fact should be borne in mind in setting the initial pH, so that the ultimate value may coincide with the desired pH. Hence the initial pH value should be fixed at 8. After the titration when the alkali is added, the broth becomes turbid again. For this reason, it should be steamed for half an hour. When cooled, it is filtered and some more distilled water is added to make it upto 1,000 c.c. During this process the original pH value falls to about 7·8. After tubing when the medium is finally sterilised in the autoclave, the pH value falls again, thus reaching a figure of about 7·6.

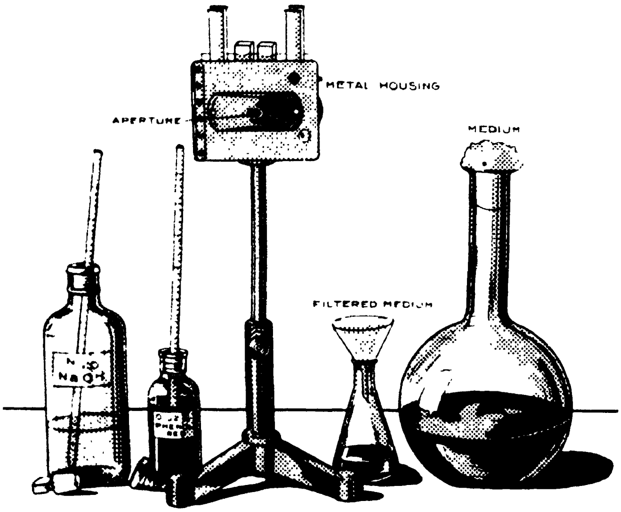


Fig. 44.

Hellige comparator set up for the adjustment of reaction of the medium.

(iv) **Filtration:**—As mentioned just now, after titration, the media are cleared by filtering through filter paper (p. 53).

(v) **Tubing of media:**—The medium is then placed in a large glass funnel fitted to the discharging glass tube by a short piece of rubber tubing provided with a thumb cork. The plug is removed from the sterile test tube of 5 × ⅝ inch. size and the glass outlet thrust deeply into the test tube (Fig. 45). About 5 to 10 c.c. are distributed in each tube and plugged with cotton wool. Care should be taken to prevent the medium from touching the upper portion of the tube, otherwise the cotton wool plugs will adhere to the wall of the tubes. For making blood cultures, larger quantities of from 75 to 100 c.c. of the broth are put up in flasks and plugged with cotton wool.

(vi) **Sterilisation:**—After tubing, the culture tubes are again sterilised in the autoclave at 20 lbs. pressure ([illegible]) for 20 minutes. Finally they are stocked in a covered jar and are k[illegible] vertical position.

Fig. 45.
Distribution of medium into tubes.

Uses of broth:—

(1) As a basis for the preparation of other media.

(2) As a preliminary for cultivation when the organisms are scanty in the material.

(3) For investigation of soluble toxic products of bacteria.

NUTRIENT AGAR

Composition:—

Standard broth (pH 7·8)	1,000 c.c.
Agar fibres	20 to 30 grams
Casein sodium or nutrose	10 grams.
White of egg (hen's)	One

Technique:—

(i) Making of the media.
(ii) Filtration.
(iii) Distribution of media.
(iv) Sterilisation.
(v) Setting of media.

(i) **Making of the media:**—The above substances are put in a flask, plugged tightly with cotton wool and the mouth is wrapped up in paper and cloth securely so that the agar fibres may not come out. This is now autoclaved for half an hour at 20 lbs. pressure (120°C). Casein sodium or nutrose is added to the broth for enrichment purpose and the white of egg for clearing the medium.

(ii) **Filtration:**—After autoclaving, the medium is filtered through cotton wool (see page 52). The filtrate is now measured and if the volume is less than the original, more distilled water is added to make it up to 1,000 c.c.

(iii) **Distribution of media:**—After filtration, the medium is either stocked in bulk or put up in test tubes of 6″ × $\frac{3}{4}$″ sizes in the same way as described for nutrient broth. The following are the approximate quantities of the medium required for various purposes:—

Sloped tubes	*7 c.c.*
Upright tubes (for plate culture)	*20 c.c.*
Deep upright tubes	*15 c.c.*

(iv) **Sterilisation:**—After distribution, the tubes are sterilised in the autoclave for 20 minutes at 20 lbs. pressure (120°C).

(v) **Setting of medium:**—In order to obtain the agar slopes for cultural purposes, the finished medium is allowed to set in a slanting position, as shown in Figure 46. The inclined position will be such as to enable the medium to occupy half the length of the test tube. After setting, the tubes are stocked in a vertical position in a covered jar which may be kept in a cool dark place, preferably in the cold chamber.

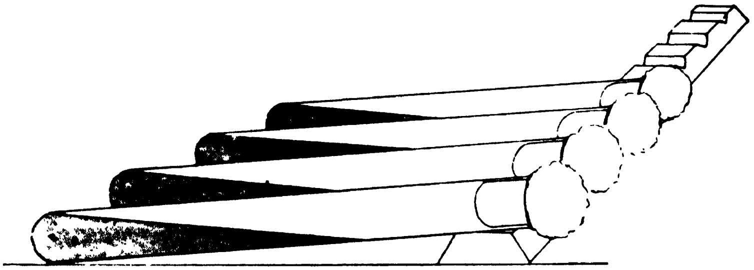


Fig. 46.

Setting of the medium in an inclined plane to form slopes.

Note: - Nutrient agar may also be stocked in upright tubes which may be utilised for making agar plates. For this purpose, it is only necessary to melt such a tube and pour the medium in a sterilised Petri dish and allowed to solidify in a horizontal position.

Uses of agar:—

(1) As a routine medium for the cultivation of most of the pathogenic bacteria.

(2) For isolation of separate colonies from a mixture.

Note: Sloped tubes are used for stroke cultures, whereas the upright tubes are meant for stab cultures, shake cultures and making of plates. (Vide making of cultures).

NUTRIENT GELATIN

Composition:—

Standard broth (pH 7·8)	...	1,000 c.c.
Gelatin leaf (French gold label)	...	150 to 200 gms.

Technique:—To broth is added 15 to 20 per cent. of leaf gelatin (best French gold label or Nelson's photographic gelatin). As autoclaving robs it of its setting property, it is best dissolved by steaming for half an hour. While the gelatin is in solution, pH is adjusted to 7·6. After titration, white of one egg dissolved in 30 c.c. of distilled water is added. It is then steamed for half an hour and filtered through cotton wool. The medium is tubed, sterilised by fractional method (p. 46) for 3 days, cooled rapidly and stored in the cold chamber.

Note:—In the tropics, it is very difficult to keep the gelatin medium solid. It may however be conveniently rendered stiff by adding 10 gms. of agar to each 1,000 c.c. of the mixture, the clearing of the medium being done with white of eggs. Even then, owing to the low melting point of gelatin (24°C.), it is very difficult to control and to use gelatin medium for cultural purposes in the tropics.

Uses:—

(1) For those bacteria which grow best at atmospheric temperature and not at that of the animal body. A cold incubator at 22°C. may be employed or in the tropics the medium may be incubated at room temperature, during the Winter Season.

(2) To study the biochemical activities of certain bacteria regarding the liquefaction of gelatin.

SPECIAL MEDIA

SPECIAL MEDIA FOR COCCI

Although some of the common cocci, such as Staphylococci and Streptococci, can grow well in the ordinary basal media, there are others, such as *Diplococcus pneumoniæ, Neisseria gonorrhææ* and *Neisseria meningitidis*, which fail to grow unless the media are enriched by some animal fluid. This is because, the latter group of organisms has no saprophytic existence and they are always found pathologically in the animal tissues. By the addition of body fluids to the basal media, we try to imitate their natural environmental conditions and hence they are suitable for their growth. The animal fluids which are added for the purpose of enrichment are blood or some elements of blood (serum) or other native albuminous substances, such as ascitic, hydrocele or ovarian fluids. The following special media are commonly used.

(1) Blood smeared agar.

(2) Blood agar.

(3) Serum agar.

(4) Hydrocele or ascitic, fluid agar.

BLOOD SMEARED AGAR:—This is a very useful medium for emergency purposes and is made as follows. A few drops of blood are collected aseptically into a sterile pipette and a few drops of it are smeared on the surface of each agar slope and allowed to clot in the sloped position.

BLOOD AGAR:—

A. WITH DEFIBRINATED BLOOD:—

Composition:—

Nutrient agar (at 50° to 55°C.)	... 100 c.c.
Defibrinated blood	... 5 to 20 c.c.

Technique:—

(i) **Preparation of defibrinated blood:**—Blood is collected in a sterile flask containing some sterile glass beads or broken pieces of glass. Human, sheep's or rabbit's blood is collected aseptically and defibrinated by vigorous shaking.

(ii) **Making of the medium:**—100 c.c. of stock nutrient agar are melted in a flask and sterilised in the autoclave at 20 lbs. pressure for 20 minutes. When it is cooled down to 50° to 55°C., sterile defibrinated animal blood is added aseptically in varying proportions, from 5 to 20 per cent. The contents are mixed by rotating the flask rapidly between the hands.

(iii) **Tubing of the medium:**—The material may then be tubed and sloped or plated as desired. Tubes must be sterile and filled with strict aseptic precautions. After setting, the tubes or plates are incubated for 24 hours before use and contaminated ones are discarded.

Note: -In order to economise the material, it may be poured over saline agar slants and sloped so as to form a uniform blood agar layer over the saline agar. About 2 to 3 c.c. of blood agar are poured over saline agar slopes (5 c.c.) and allowed to set in the inclined position. The mouth of the flasks and test tubes should be flared at each step.

Preparation of saline agar:—

Normal saline	... 500 c.c.
Agar fibres	... 10 gms.

Technique:—This is exactly the same as in the preparation of nutrient agar.

B. WITH CITRATED BLOOD:—This may be used in place of defibrinated blood.

Preparation:—About 2·5 c.c. of a 5 per cent. sodium citrate solution are taken in a flask and 25 c.c. of human or rabbit's blood are added to it. The contents are mixed thoroughly to prevent coagulation by rapidly rotating the flask, the resulting mixture containing about 0·5 per cent. sodium citrate. This citrated blood will keep in good condition in a cool place, for about a week. As the cells will sink to the bottom, the flask should be well shaken before use.

Uses of blood agar:—

(1) For the growth of many delicate microbes which do not grow in ordinary media, e.g., D. pneumoniæ, N. gonorrhœæ and N. meningitidis.

(2) For the detection of hæmolytic property of many bacteria.

(3) For the differentiation of various strains of some organisms, e.g., Streptococcus.

SERUM AGAR:—

Composition:—

Melted nutrient agar	...	300 c.c.
Sterile serum (uncoagulated)	..	50 c.c.

Note:—The medium contains about 14 per cent. serum.

Technique:—

(i) **Collection of blood serum:**—A convenient receptacle is a large enamelled pail with a lid or a glass basin, as shown in Figure 47, which should be sterilised in the autoclave. Blood should be collected, with as much asepsis as possible, from a slaughter-house. The vessel should be two-thirds full and kept in the refrigerator or ice-chest overnight. The adhering clots may be freed from the sides and the vessel should be allowed to remain undisturbed either on the table or in an incubator. The separated serum may be used at once for making media or stored for future use with 0·25 per cent. chloroform or 10 per cent. ether, in tightly corked flasks in the cold storage. If the serum obtained has a red colour indicating the presence of red blood corpuscles, the latter can be removed by centrifugalisation and the clear serum carefully secured. Before using the serum the preservative may be driven off by heating at 45°C.

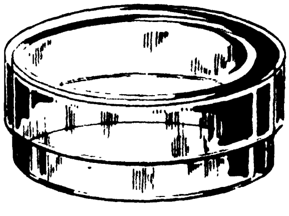


Fig. 47.

Glass basin for collecting blood serum.

(ii) **Preparation of serum agar:**—Stock nutrient agar is melted in an autoclave and when it is cooled down to 50° to 55°C., it is kept in a water bath at 50°C. With a sterile measuring pipette, the required amount of serum is added to it gradually. The mixture is shaken and distributed aseptically in the usual way and sloped or plated as desired. As this medium is indistinguishable from ordinary nutrient agar, all tubes or plates should be carefully labelled. Tubes are incubated before use to avoid contamination.

Uses of serum agar:—Same as that of blood agar.

HYDROCELE OR ASCITIC-FLUID AGAR:—These are made in the same way as serum agar. To tubes of melted nutrient agar (3 per cent. agar) at 50°C., 1 to 3 c.c. of hydrocele or ascitic-fluid are added aseptically. Slopes or plates are then made with the same precaution as in blood or serum agar. Tubes are incubated before use.

Collection of fluid:—These are collected aseptically with usual precautions from operation theatres. They are stocked in a cold chamber. Ascitic fluid varies greatly in reaction and composition and on the whole it is not so equally good as the hydrocele fluid.

Uses:—These are particularly used for the cultivation of N. gonorrhœæ and N. meningitidis.

MEDIA FOR CORYNEBACTERIUM DIPHTHERIÆ

1. **ORDINARY SERUM MEDIA:**—After collecting the sterile serum in the way described before, it is distributed in 5″ × ⅝″ sterile test tubes. About 5 c.c. of the sterile serum are put into each tube aseptically by means of a sterile pipette (Fig. 48). They are then placed in the serum inspissator in an inclined position (so as to obtain the desired slope) and sterilised by fractional or intermittent method, i.e., on the first day they are exposed to 80°C. for 1 hour, on the second day to 75°C. for half an hour and on the third day again to 75°C. for half an hour. By this process, sterilisation and inspissation are carried out at the same time. The media are incubated before use.

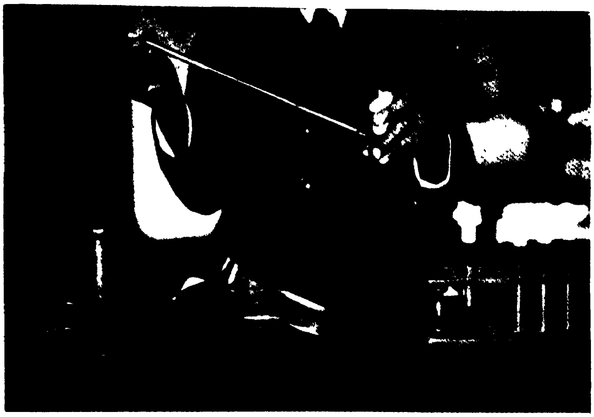


Fig. 48.

Preparation of serum medium. Serum is pipetted aseptically from the glass basin into test tubes.

2. **LOEFFLER'S SERUM MEDIUM:**—

Composition:—

Sterile standard broth with 2 per cent. glucose ...	100 c.c.
Uncoagulated serum from ox, sheep or horse ...	300 c.c.

Technique:—The serum is collected in the usual way and the above constituents are put in a sterile flask of 500 c.c. capacity. They are mixed thoroughly and may be warmed, so as to remove the chloroform from the stock serum. About 5 c.c. of the medium are taken out by means of a sterile pipette and distributed aseptically into 5″ × $\frac{5}{8}$″ sterile test tubes which are put in the serum inspissator for sterilisation and inspissation in a sloped position.

3. **SERUM AGAR:**—This is an excellent medium for the growth of C. diphtheriæ. For preparation see page 62.

4. DORSET'S EGG MEDIUM:—

Composition:—

Fresh hen's eggs	... 4
Distilled water	... 24 c.c.

Technique:—

The eggs are thoroughly washed under tap water and then placed in a saturated watery solution of corrosive sublimate for 15 minutes. They are washed again in running tap water. The shells are then sterilised with absolute alcohol after which the eggs are broken into a sterile wide mouthed glass beaker, handling them with sterile rubber gloves. For each egg, 6 c.c. of distilled water are added and thoroughly mixed by vigorous shaking until a thin paste is formed. The mixture is then filtered through sterile cloth and the filtrate is distributed aseptically in quantities of 5 to 7 c.c. into sterile test tubes, the mouths being flared at each step. They are then sloped and sterilised in the serum inspissator by fractional method.

5. **TELLURITE MEDIUM** (of Douglas, Allison and Ayling).—This is a selective and indicator medium.

Composition:—

Serum (ox or horse)	... 100 c.c.
Liquor trypsin Co. (Allen & Hanbury's)	... 8 c.c.
2 per cent. aqueous solution of potassium tellurite (British Drug House)	... 10 c.c.

This mixture is allowed to stand in the cold for 24 hours and sterilised by filtration through a Seitz filter and stocked.

Preparation of the medium:—

Nutrient agar, melted and cooled to 50°C.	... 100 c.c.
Tellurite mixture (stocked as above)	... 10 c.c.
10 per cent. copper sulphate solution (B. D. H.)	... 0·5 c.c.

The above are put in a sterile flask and after thorough mixing, are distributed as slopes or plates in the usual way. This medium is selective for Diphtheria and Diphtheroid bacilli, the growth of other bacteria of the throat being inhibited.

MEDIA FOR MYCOBACTERIUM TUBERCULOSIS

1. **GLYCERIN BROTH:**—6 to 8 per cent. glycerin (specific gravity 1·25) is added to filtered nutrient broth and sterilised by fractional method.

2. **GLYCERIN AGAR:**—5 per cent. glycerin (Sp. gr. 1·25) is added to filtered nutrient agar and sterilised by fractional method.

3. **GLYCERIN EGG MEDIUM:**—One part of saline glycerin (6 per cent. glycerin in normal saline) is added to three parts of beaten eggs. The latter are prepared in the way as described for Dorset's egg medium.

4. **GLYCERIN POTATO MEDIUM:**—Potatoes are used as slices in tubes. A large, long potato is well washed, scrubbed thoroughly with a brush under the tap, dried and peeled with a clean knife. Cylindrical slices of ¾" diameter are cut off by means of a large cork borer or an apple corer and are divided obliquely (Fig. 49.A); the diseased or bruised parts are rejected. The slices are soaked in water over-night and are then placed in 6 per cent. glycerin in saline for one hour. After this each piece with its thick end at the bottom is placed in a special test tube, such as Ehrlich's or Roux's (Fig. 49B), with about 5 c.c. of glycerin and subjected to steaming for 30 minutes.

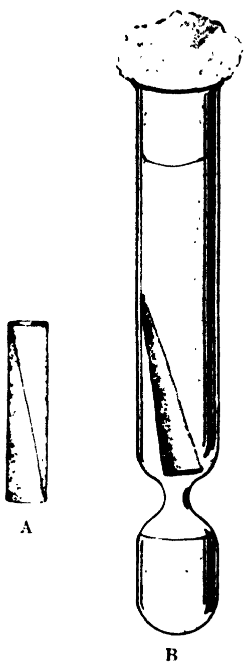


Fig. 49.

A Cylinder of potato cut obliquely; B - Roux's tube with a piece of potato and glycerin solution at the bottom.

5. **DORSET'S EGG MEDIUM:**—This is the same as that used for C. diphtheriæ. The medium may be modified by the addition of 6 per cent. glycerin in normal saline (0·85 per cent.) in place of distilled water.

6. **PETROFF'S MEDIUM:**—

Composition:—

Glycerinated meat juice	...	400 c.c.
Egg filtrate	...	200 c.c.
Alcoholic solution of gentian violet (1 per cent.)	...	6 c.c.

Mix well and distribute in 6" × ¾" test tubes. Sterilise in sloped position in the serum inspissator by fractional method.

Technique:—

(i) **Preparation of glycerinated meat juice:**—250 grams of fresh meat (beef or veal) are finely minced and mixed with 212 grams of distilled water and 37·5 grams of glycerin in a sterile fl [illegible] The whole is placed in the ice-chest overnight and is then filtered thr [illegible] rile gauze.

(ii) **Preparation of egg filtrate:**—16 [illegible] ggs are sterilised by immersing them in a vessel containing 70 per [illegible] ohol, the whole being placed in a water-bath at 70°C. for 15 minut [illegible] eggs are then broken into a sterile flask, handling them with sterile rubber gloves. The white and yolk of eggs are thoroughly beaten up by shaking and filtered through a sterile gauze.

(iii) **Preparation of alcoholic solution of gentian violet:—**

Gentian violet	... 0·5 gm.
Alcohol 95 per cent.	... 50 c.c.

To every 100 c.c. of the mixture of egg filtrate and glycerinated meat juice, 1 c.c. of the solution is added so as to make a final concentration of 1 : 10,000.

MEDIA FOR COLI-TYPHOID-DYSENTERIÆ GROUP OF ORGANISMS

Numerous media are used for this group of organisms. Some are useful for routine diagnostic work, while others are of interest to those who work on special lines, such as the bacteriological study of water, milk, sewage, etc.

1. **MACCONKEY'S AGAR MEDIUM:**—(Neutral-red, bile-salt, peptone, lactose, agar).

Composition:—

Peptone (Witte's)	... 20 gms.
Sodium taurocholate	... 5 gms.
Tap water	... 1,000 c.c.
Agar fibres	... 25 to 30 gms.
Casein sodium or nutrose	... 10 gms.
White of egg	... One
Lactose (pure)	... 15 to 20 gms.
Neutral red, 1 per cent. solution	... 5 c.c.

Technique:—

(i) In a flask 1, 2 and 3 are taken and steamed for half an hour. PH is then adjusted to 7·8 and after adding the required alkali, the mixture is steamed and filtered.

(ii) To the above, the following substances are then added, agar, nutrose and white of an egg and autoclaved at 120°C. for half an hour.

(iii) It is then filtered through cotton wool and the filtrate measured; if necessary, more distilled water is added to make it up to 1,000 c.c.

(iv) To the filtrate, lactose in solution (1·5 to 2 per cent.) and freshly prepared aqueous solution of neutral red (1 per cent.) are added.

(v) The medium is then sterilised in a steam steriliser by fractional method, i.e., exposed to 100°C. for half an hour each day on three successive days and stocked in 200 c.c. flasks.

As required, the stock medium is melted in a steam steriliser and when the temperature comes down to 60°C., it is poured in sterilised plates and allowed to set in a horizontal position. Before use the media are dried by keeping them upside down in the incubator with the lids half open for 2 to 4 hours.

2. ENDO'S MEDIUM:—

Composition:—

A. Of stock medium:—

Heart extract	... 1,000 c.c.
Peptone	... 10 gms.
Sodium chloride (chemically pure)	... 5 gms.
Agar fibres	... 25 to 30 gms.
Casein sodium or nutrose	... 10 gms.
White of egg	... One

B. Of actual medium:—

Stock medium (melted)	... 200 c.c.
Lactose	.. 2 to 3 gms.
Alcoholic solution of basic fuchsin (10 per cent.)	... 0·8 c.c.
Sodium sulphite (10 per cent. solution)	... 5 c.c.

Technique:—

(i) **Preparation of stock medium:**—1, 2 and 3 are mixed in a flask and steamed for half an hour. PH is adjusted at 8 to 8·2, i.e., deeply alkaline to litmus paper. After adding the required alkali, it is steamed again and then filtered. The filtrate is measured and made up to 1,000 c.c. To the filtrate are now added 4, 5 and 6 and the whole is autoclaved at 115°C. for half an hour. The mixture is then filtered through cotton wool and the filtrate made up to 1,000 c.c. and stocked.

(ii) **Preparation of the actual medium:**—The stock medium is melted and to 200 c.c., the following are added and mixed thoroughly by shaking:—

- (a) 3 gms. of pure lactose solution—this is sterilised by fractional method.
- (b) 0·8 c.c. of a 10 per cent. alcoholic solution of basic fuchsin.
- (c) 5 c.c. of a 10 per cent. solution of sodium sulphite (freshly prepared) in sterile distilled water.

(iii) The medium is then distributed in sterile plates aseptically and the finished product is stocked in the ice-chest. The medium is of pink colour when hot but becomes almost colourless when cold.

Note:—Endo's medium is not very suitable in the tropics, as when exposed to the room temperature and light, the sulphite is reduced and the medium becomes red. Moreover, the colour of the colonies in this medium tends to diffuse.

3. LACTOSE-LITMUS AGAR:—

Composition:—

Nutrient agar (melted)	200 c.c.
Lactose (pure)	3 gms.
Litmus solution	q. s.

Technique:—

(i) To 200 c.c. of melted nutrient agar are added 3 gms. of lactose and sufficient litmus solution to obtain a decided blue colour.

(ii) The medium is then tubed in 10 c.c. and sterilised by steaming by the intermittent method.

(iii) It is then poured in sterile Petri dishes and plated. Before use the medium is dried in the incubator.

4. BROM-CRESOL PURPLE MEDIUM:—

The preparation is exactly the same as that of litmus lactose agar, with the exception that the indicator used is 1 per cent. solution of brom-cresol purple, instead of litmus solution. This medium is particularly suitable for the growth of cocci, specially the Gram-positive ones, which are found in stools.

SELECTIVE MEDIA FOR EBERTHELLA TYPHI (B. TYPHOSUS)

1. WILSON AND BLAIR'S MEDIUM (BISMUTH-IRON AGAR):—

Composition:—

Hot sterile agar (pH 7·0)	100 c.c.
20 per cent. sterile glucose	5 c.c.
Bismuth-iron stock solution	16 c.c.
1 per cent. sterile brilliant green solution	0·5 c.c.

The mixture is poured into plates and dried before use.

Preparation of Bismuth-iron stock solution:—

(This can be kept in the laboratory for over a month).

20 per cent. anhydrous sodium sulphite	100 c.c.
Liquor Bismuthi	50 c.c.
8 per cent. solution of ferrous sulphate crystals	100 c.c.
10 per cent. solution of desiccated sodium phosphate	100 c.c.

Mix and boil together.

Liquor Bismuthi is prepared as follows:—

Dissolve 60 gms. of bismuth citrate (made into a thin paste with 50 c.c. distilled water) in 20 c.c. liquor ammonia and make up to 500 c.c. with distilled water.

2. BRILLIANT-GREEN PEPTONE WATER:—

Composition:—

(i) Peptone water (Peptone 2 per cent., sodium chloride 0·5 per cent.)	10 c.c.
(ii) Brilliant green, 1 in 10,000	0·25 to 0·7 c.c.

An aqueous solution of brilliant green, 1 in 10,000, is prepared and sterilised in the autoclave. As varying concentrations of brilliant green give better results, 0·7, 0·4 and 0·25 c.c. of the above are added to three tubes of peptone water (10 c.c. each). Each tube is then inoculated with a loopful of the fæces and after 24 hours' incubation, is plated on MacConkey's medium.

Note:—It has been found out later that although the brilliant green inhibits the growth of Escherichia coli, it has no effect on Aerobacter ærogenes (B. lactis ærogenes) which appears to be equally resistant as E. typhi. Hence it has been recommended that 0·4 c.c. of 1 in 1,000 telluric acid solution should be added to the brilliant green peptone water which inhibits the growth of both E. coli and A. ærogenes but not that of E. typhi.

3. CONRADI'S BILE MEDIUM:—

Composition:—

Ox bile	... 90 c.c.
Glycerin	... 10 c.c.
Peptone	... 2 grams.

Mix well and tube. Give fractional sterilisation for 30 minutes for three consecutive days. This medium is commonly used for blood cultures in enteric cases.

SPECIAL MEDIA FOR VIBRIO COMMA (VIBRIO CHOLERÆ)

1. DIEUDONNÉ'S BLOOD-ALKALI AGAR:—

Composition:—

Equal parts (500 c.c. each) of defibrinated blood and normal (N/1) NaOH solution are mixed and sterilised for half an hour by live steam. By the action of the alkalis, ammoniacal substances are volatilised, so the steaming should be repeated till no ammoniacal smell is given off. Three parts (300 c.c.) of this mixture are now added to seven parts (700 c.c.) of a 2 to 3 per cent. nutrient agar (neutral to litmus) and thoroughly mixed.

This blood-alkali agar is poured into plates and kept in the incubator overnight half open. Next morning the lid is closed. The medium is now ready for use.

2. DUNHAM'S PEPTONE-SALT SOLUTION:—

Composition:

Peptone	... 10 gms.
Sodium chloride (C. P.)	... 5 gms.
Distilled water	... 1,000 c.c.

The ingredients are dissolved by boiling, cooled and filtered till clear. PH is adjusted to 8 and after titration steamed again and then filtered through filter paper. It is then distributed [illegible] quantities of 5 to 7 c.c. in each tube and autoclaved at 120°C. for 20 minutes.

3. TAUROCHOLATE AGAR:—

Composition:—

Nutrient agar	... 100 c.c.
Sodium taurocholate	... 1 gm.

The mixture is dissolved by steaming for half an hour and filtered if necessary. It is then sterilised in the autoclave and plated as usual.

MEDIA FOR HÆMOPHILIC GROUP OF BACTERIA

Media for H. influenzæ

1. BOILED BLOOD AGAR (CHOCOLATE AGAR):—

Composition:—

Nutrient agar (at 60°C.)	... 100 c.c.
Defibrinated blood (Rabbit's)	... 5 c.c.

Technique:—Stock nutrient agar is melted and when the temperature is reduced to 60°C., 5 c.c. of defibrinated blood are added to it. The mixture is then heated and the temperature brought up to boiling point (100°C.). The medium when cooled to 50° to 60°C., is distributed aseptically into sterile test tubes in quantities of 5 to 7 c.c. each, and allowed to solidify in the sloped position. Plates may be prepared by pouring the required amount in Petri dishes.

Use:—This medium is used for mass cultures of influenza bacillus.

2. PEPTIC BLOOD AGAR (FILDES):—

Composition (Preparation of digest-blood):—

Normal saline	... 150 c.c.
Concentrated HCl	... 6 c.c.
Defibrinated sheep's blood	... 50 c.c.
Pepsin, B. P. granulated	... 1 gm.

Technique:—

The above ingredients are put in a 250 c.c. sterile glass-stoppered bottle and dissolved by shaking. The bottle is then placed in a water-bath at 55°C. for 4 hours. After this, 12 c.c. of 20 per cent. NaOH are added. The reaction is then tested with cresol-red by removing ½ c.c. with a pipette and diluting 1 in 5 with distilled water. The addition of cresol red will produce a permanganate colour and until this is obtained, more NaOH is to be added. Concentrated hydrochloric acid is now added drop by drop by means of a sterile pipette until cresol red gives no colour and phenol red gives red, i.e., pH is adjusted to 6·9 to 7. For preservation 0·25 per cent. chloroform is added. This stock digest-blood keeps well for 12 months in slightly acid reaction.

For use:—

Nutrient agar (60°C.)	... 100 c.c.
Digest-blood	... 5 c.c.

The above are mixed and distributed aseptically into sterilised test tubes and sloped or poured in Petri dishes and plated.

Medium for H. pertussis

BORDET AND GENGOU'S MEDIUM:—

Composition:—

Potato agar (55°C.)	... } Equal parts.
Defibrinated blood (sheep's or human)	... }

Potato agar:—

Extract of potato	... One part (50 c.c.)
Saline agar (agar 4 per cent.)	... Three parts (150 c.c.)

Technique:—

(i) An extract of potato is first prepared by adding 100 gms. of sliced potato (made as described on p. 65) to 200 c.c. of a 4 per cent. aqueous glycerin. The mixture is steamed for 30 minutes and strained through gauze.

(ii) To one part of this potato extract (50 c.c.) are added three parts (150 c.c.) of 0·6 per cent. saline. Then 6 gms. of agar (4 per cent.) are added to the mixture. They are shaken thoroughly, steamed for half an hour and filtered. The filtrate forms the potato agar.

(iii) 2 to 3 c.c. of potato agar are distributed in test tubes and sterilised by steaming. After the temperature is reduced to 55°C., an equal volume of defibrinated blood (p. 61) warmed to 40°C. is added and uniformly mixed. The medium may then be allowed to set in slopes or plates.

MEDIA FOR BIOCHEMICAL REACTIONS

Fermentation tests:—These are very valuable for identifying and differentiating the individual bacterial species within a particular group. The substances commonly employed are:—

1. *Sugars (a) Mono-saccharides—(i) Pentoses; Xylose; arabinose.*
(ii) Hexoses; Glucose (dextrose), fructose (Lævulose), galactose.
(b) Di-saccharides—Lactose; sucrose (saccharose) and maltose.
(c) Tri-saccharides—Raffinose.
(d) Poly-saccharides—Dextrin, inulin and starch.

2. *Alcohols—Glycerol, dulcitol, mannitol, etc.*

3. *Glucosides—Salicin, æsculin, etc.*

Media used for fermentation tests are—(i) Sugar media, (ii) Hiss's serum water media and (iii) glucose agar.

Sugar media

Composition:—

Peptone	1 gm.
Sodium chloride	0·5 gm.
Distilled water	100 c.c.
Sugar	2 gms.
Aqueous solution of acid fuchsin (0·5 per cent.)	

Technique:—

(i) 1, 2 and 3 are mixed in a flask and dissolved by steaming. Reaction is adjusted to pH 7·4 to 7·6. After titration, the mixture is steamed again and then filtered.

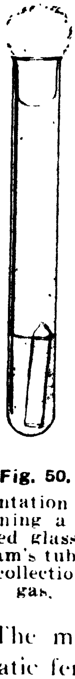


Fig. 50. Fermentation tube containing a small inverted glass tube (Durham's tube) for the collection of gas.

(ii) To the filtrate are added 2 gms. of sugar and 1 c.c. of fuchsin. Instead of fuchsin other indicators may also be used.

(iii) About 3 to 5 c.c. of the medium are then distributed in sterile test tubes, containing Durham's gas tubes. The latter consist of small inverted test tubes which have been slipped down into the culture tubes for observation of gas formation (Fig. 50).

(iv) The tubes are then sterilised by fractional method. The air remaining in the smaller tubes is thereby driven out.

Note:—Different sugar media may be conveniently recognised by the use of cotton wool plugs of different colours or leaving different coloured sterile glass beads at the bottom of the sugar tubes.

Hiss's serum water medium

Composition:—

Clear beef serum	1 part.
Distilled water	3 parts.

The mixture is heated for 15 minutes in live steam to destroy any diastatic ferment that may be present. If necessary, it may be filtered. It is then coloured with litmus solution until a clear transparent blue colour is obtained. Then 1 to 2 per cent. of any desired sugar is added and sterilised by the fractional method.

Use:—Acid fermentation is shown by the change in reaction and the resulting coagulation and gas production by bubbles in the coagulum. It is useful for delicate organisms, e.g., C. diphtheriæ, N. meningitidis, Streptococci, and D. pneumoniæ as sugar media with broth or peptone water do not give good results owing to poor growth.

When inulin medium is prepared, the required amount is added to a small quantity of distilled water and sterilised in the autoclave at 15 lbs. for half an hour. It is cooled and then added to the serum. After this, it is coloured with litmus, tubed, and given fractional sterilisation for 20 minutes for 3 consecutive days. If levulose is the desired sugar, four to five parts of sugar solution are added to one of the serum since this particular sugar has a tendency to coagulate the serum.

Glucose agar

Composition:—

Nutrient agar (melted) ...	100 c.c.
Glucose (Pure) ...	2 gms.

The above are dissolved by steaming for 20 minutes, distributed in test tubes, and then sterilised by fractional method. The medium is inoculated in the melted condition and then allowed to set in a sloped or vertical position. Formation of gas is shown by breaking up of the medium or the collection of gas bubbles in it. If an indicator is used in the preparation of the medium, change in its colour will show the acid production. This medium is also used for the cultivation of anaerobic organisms.

Litmus milk

This is useful for observing the effect of bacterial growth on milk, *e.g.*, production of acid by fermentation of lactose of the milk, and coagulation of the soluble albumin.

Preparation:—Fresh milk (200 c.c.) is centrifugalised to separate the cream, then heated to boiling point and left overnight in an ice-chest. The fat-free milk is pipetted off, coloured deeply with litmus and tubed. Fractional sterilisation should be given for 30 minutes for three consecutive days.

Indol production

The medium commonly used is Dunham's peptone-salt solution of pH 7·4 and indol formation is determined by the following reactions:—(a) Nitroso-indol reaction. (b) Ehrlich's test (most delicate).

Medium for Methyl Red and Voges Proskauer test (Buffered glucose broth)

Composition:

Peptone ...	5 gms.
Glucose ...	5 gms.
Dipotassium-hydrogen phosphate (K_2HPO_4) ...	5 gms.
Water ...	800 c.c.

Technique:—

The above substances are dissolved by heating, filtered, cooled and the volume made up to 1,000 c.c. Each tube is t[illegible] [illegible]lled up with 10 c.c. of the medium and sterilised by fractional method f[illegible] [illegible] days.

Medium for Koser's test (citrate medium)

Composition:—

Sodium ammonium phosphate	... 1·5 gms.
Potassium dihydrogen phosphate	... 1·0 gm.
Magnesium sulphate	... 0·2 gm.
Sodium citrate (crystals)	... 2·5 gm.
Water	... 1,000 c.c.

The above is then distributed into tubes and sterilised in the autoclave.

Medium for Uric acid test

Composition:—

Sodium Chloride	... 5 gms.
Magnesium sulphate	... 0·2 gm.
Calcium chloride	... 0·1 gm.
Dipotassium-hydrogen phosphate (K_2HPO_4)	... 1 gm.
Glycerin	... 30·1 gm.
Uric acid	... 0·5 gm.
Distilled water	... 1,000 c.c.

The above are dissolved in a flask, sterilised in the steam steriliser and tubed as usual.

Medium for the detection of H_2S production (Lead-acetate-agar)

Preparation:—A 10 per cent. watery solution of basic lead acetate is made and sterilised in the autoclave. 1 c.c. of this is added to 200 c.c. of melted nutrient agar cooled to 60°C. The final strength of the salt is 0·05 per cent. The medium is then distributed aseptically as upright tubes and is inoculated by stabbing.

Use:—For differentiating Salmonella group of organisms.

CHAPTER VI

MAKING OF CULTURES

GENERAL REMARKS:—

1. All culture tubes should be used as fresh as possible.

2. After their preparation, culture media should be kept in a cool dark place and well covered, so as to prevent evaporation of moisture and also to protect them from dust and contamination.

3. The top of the tubes may be protected by rubber caps or covered with paper and tied with thread. In the case of agar slants or similar media, the tubes should always be kept in a vertical position. The water of condensation should not soak the cotton plug.

4. In the case of solid media, there should be some moisture at the bottom of the tube, as water of condensation. A dry medium is most unsuitable for the cultivation of important and delicate micro-organisms and should always be discarded. Unless this point is kept in mind, much discrepancy may occur in the report on the bacteriological examination of pathological materials.

Culture tubes:—They should always be held during use, as horizontally as possible. This prevents micro-organisms which are always present in the atmosphere from falling directly on the surface of the medium and thus contaminating it. Whatever bacteria are deposited about the mouth of the test tube, may subsequently be destroyed by heating the part sufficiently over the flame. Cotton plugs should be removed very carefully and just before use. The plug should first be burnt in the flame which may then be extinguished by quickly pressing it in the palm of the hand. This procedure destroys all micro-organisms which might have been on the cotton plug and which might otherwise contaminate the culture. Before actually removing it, the cotton plug should be twisted well in the test tube so as to break away all adhesions. Unless this precaution is taken, a very considerable number of cotton fibres may stick out of the mouth of the tube and increase the risk of contamination. The next step is to heat the upper end of the tube, including the rim, over the flame to sterilise the part. After the medium is inoculated, the bottom of the plug should be burnt over the flame and then replaced into the tube as rapidly as possible.

During inoculation, the culture tube should be held as slantingly as possible in the left hand, preferably between [illegible] thumb and the tips of the other fingers and the cotton plug grasped [illegible] the little finger of the right hand. When more than one tube [illegible] be handled, as when making subcultures, both the tubes are held in the left hand and the plugs grasped between the fingers of the right hand near their webs (Fig. 51). The inner portions of the plugs should be held away from the palm.

As direct sun-light is always injurious to bacterial life, the culture tubes after inoculation should never be left outside on the table, but kept inside the incubator at once. Every culture tube should be labelled or marked, regarding the name, strain, date and other details before it is put inside the incubator.

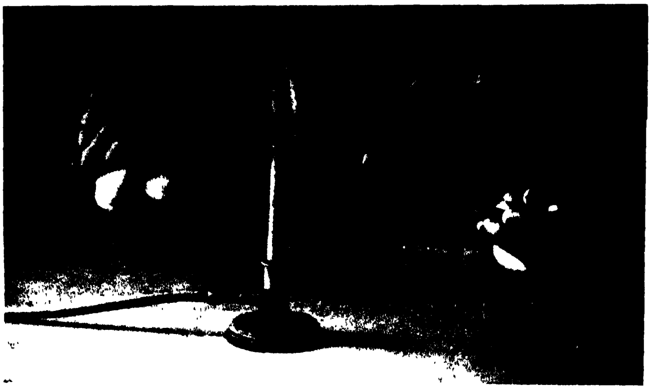


Fig. 51.

Method of inoculating sloped tubes one from the other. Note how the platinum loop, tubes and plugs are held.

Platinum loop (Fig. 52):—The wire should not be too thin nor too thick. The length should be 6 to 8 cm. and may be mounted on a piece of glass rod. A loop is very conveniently made by moulding the free end of the wire around a thin glass rod. The loop should be complete and about 0·5 cm. in diameter. Unless it is complete and closed, it will not carry drops of the material, specially when dealing with a liquid culture. The handle of the platinum loop should be held like a pen in the right hand. Before using it, the wire is heated red-hot in the flame, taking care that the adjacent portion of the holder is also heated and thus fully sterilised. It should then be allowed to cool, preferably in the water of condensation or by touching that part of the medium which is free from growth. This is very important, otherwise the hot wire may kill the bacteria which are touched with it.

Fig. 52.

Platinum loop in glass handle for making stroke culture.

Inoculation of media:—During inoculation of a **solid medium,** the loop containing the material is gently rubbed on the surface of the medium, taking sufficient care not to dig in to the substance. When the inoculation is over, the wire should again be thoroughly sterilised before being kept on the table. The worker should pay attention to a few little details about the use of the platinum wire. Either during use or during sterilisation in the flame, the wire should always be held with the loop directed downwards and the holder directed upwards. Unless this is done, a liquid culture may trickle down the wire on the holder and contaminate the hand of the worker. When the loop contains a large quantity of bacterial culture, either liquid or solid, it should first be held, during sterilisation, a little distance above the flame and then slowly and gradually lowered and directly brought in contact with the flame. Unless this is done, the too sudden heating in a strong flame may cause much spurting of the material, leading to contamination of the table and even the worker's clothes.

The mode of inoculating a **liquid medium** is very simple. The charged platinum loop is immersed into the medium and gently shaken or the loop is rubbed on the inner side of the test tube at a level just a little above the top of the fluid which is then intimately mixed with it.

In making a **deep stab culture** in a solid medium, a perfectly straight platinum wire (Fig. 53) is necessary. The wire is charged with the required culture and gently plunged deep into the centre of the medium. When dealing with an anaerobic organism, the top part of the medium may be heated till it melts and then allowed to cool so that the needle track, which might allow air to go in, becomes obliterated and a condition of anaerobiosis obtained.

Fig. 53.

in glass handle for making stab culture.

It must be remembered as a common bacteriological principle that whenever any material is to be cultured, a very small quantity, not more than a loopful, is required. The smaller the quantity of material taken, the better and more discrete are the colonies obtained and this always facilitates study. In the case of a primary culture, it is better to spread the material on the surface of an agar tube and then without recharging, carry it into another and, if necessary, into a third. If the number of bacteria is not large, discrete colonies may be obtained in the first. But if they are very numerous, the first tube may have to be discarded and it may be the second or even the third tube which will show discrete colonies. If economy in culture medium is wanted, the required material may be emulsified in a little sterile saline and a loopful from the latter may then be spread on the surface of an agar slope or plate. A very convenient practice followed by the writers is to stain a smear of the material and examine it under the microscope, so as to obtain an approximate idea about the bacterial content before doing the cultural work.

When it is necessary to obtain the gro[illegible]f an organism in a pure state from a mixture of two or more, soli[illegible] and specially, selective indicator (differentiating) media are used in [illegible]s known as Petri dishes. A culture medium spread on a Petri dish is [illegible] plate and the process of culturing bacteria on such media is called the **plating method.** The bacteria are distributed over a larger area and there is every chance that the individual bacterium may be scattered so far away from each other as to be able to form single discrete colonies from which they may be easily picked up. A Petri dish (Fig. 54) consists of two flat glass plates with rims about half an inch raised. One of them is smaller and the other slightly bigger, so as to be able to cover the small one, for which it is commonly called the lid. The requisite medium is spread as a thin layer on the smaller plate.

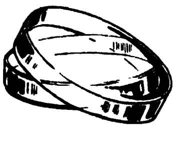


Fig. 54.

Petri dish for making "plates"; the lid is shown partially raised.

Methods of inoculation of plates:—The actual inoculation may be done in one of the following ways:—

(i) *Spreading or streaking method:*—By this method a small amount of material for examination is taken either with a sterile platinum loop or a capillary pipette and placed on the medium in the plate (Fig. 55A). The next step consists in spreading it uniformly on the entire surface of the medium. There are various ways of doing this, each worker having his own personal liking. The material may be spread with a platinum loop in streaks or lines, across the plate, without recharging the loop with fresh material. Some workers prefer to draw one set of parallel lines in one direction followed by another set cutting the former at right angles (Fig. 55B & C). It is customary to find continuous lines of growth along the first two or three streaks but fairly discrete colonies develop along the others.

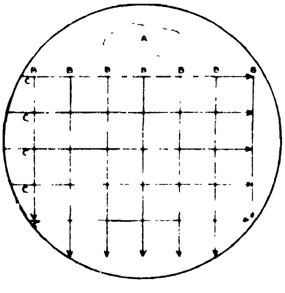


Fig. 55.

Method of inoculating a surface plate with a platinum loop.

A The area over which the inoculating material is placed.

B & C Indicate the line of strokes made without recharging the loop.

A much better method of spreading the material on the plate is to use a *spreader*. This usually consists of a thin glass rod or a narrow pipette bent at one end over a Bunsen flame the forms sh in Figure 56. This is first sterilised over a flame and then allowed to cool. Before use, it should first touch the water of condensation on the lid, if there be any, or the surface of the medium not containing the material for examination. Unless this little point is remembered, a spreader may remain much too hot when it is used for spreading purposes and thus most of the bacteria may be killed and a negative or an erroneous report may be made about the bacterial content of the suspected material. The drop of material at one edge is first well rubbed with the spreader on a small surface of the plate. Then the

Fig. 56.

Spreader made from a Pasteur pipette.

spreader is lifted and rubbed over another area. In this way the process is repeated several times till all the surface of the plate is covered, sufficient care being taken not to rub the same surface twice (Fig. 57). If the material contains a very large number of organisms, the surfaces of two plates may be used. In this way, excellent distribution and very good discrete colonies are obtained.

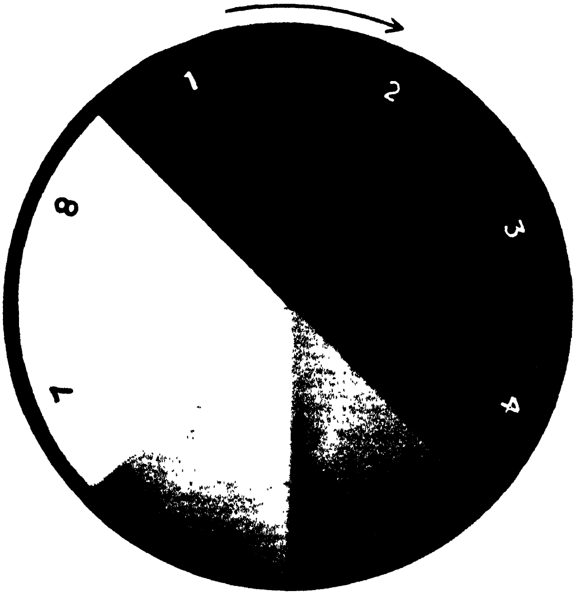


Fig. 57.

Method of inoculating a plate with a spreader. The arrow indicates the direction in which the plate is rotated and the figures in the shaded areas show the segments in which successive inoculations are made without recharging the spreader. The grades of shading indicate the successive distribution of the material and the resulting growth.

(ii) *Pour-plate method:*—This is used very extensively in the bacteriological examination of water. In this method, a small portion of the material is well mixed with the medium while still liquid at about 50°C. and the mixture poured into the plate and allowed to solidify. The bacteria develop deep colonies which may then be picked up for further study. In this connection, it should be remembered that agar melts at a temperature, a little lower than 100°C. and solidifies at about 42° to 44°C. The medium in the tube should be heated in a water-bath till it melts. The temperature of the bath is now lowered by the addition of cold water till it reaches near about 45° to 46°C. The temperature should be measured with a thermometer and no guess work should be allowed in this matter. Because, if the material is added to the melted medium at a high temperature, the organisms may be killed and if the temperature is lowered too low, before the material is added, one may not be able to mix it thoroughly and pour it into the Petri dish, owing to rapid solidification. Any way, [illegible] required material in suitable quantity and dilution is mixed with the me[illegible]dium at the proper temperature by rapidly rotating the tube betwee[illegible]o hands. The cotton plug being flamed and the top of the test tube being sterilised by heating on the flame, the medium is gently poured into the dish by raising the lid of the plate at one side just sufficiently to allow the tube to be introduced under it (Fig. 58). The lid should be replaced at once and the plate left on the table and not disturbed until the medium is cool and completely solidified.

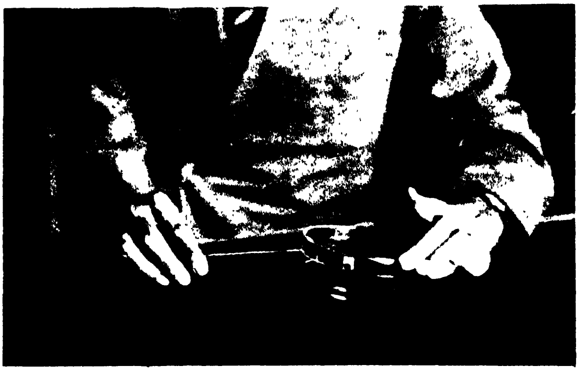


Fig. 58.

Method of "pouring" the medium in the sterile Petri dish for plate culture.

Some general facts about making plate culture:—

(1) All plates, specially those of nutrient agar, blood or serum agar, coagulated blood serum, etc. which do not contain any inhibiting reagent, should be prepared fresh before use. This has a distinct advantage. For instance, if by chance, any micro-organism falls on the surface of such a plate, it will form on incubation, only one colony which may be easily marked off. On the other hand, if such a plate is kept unused for a day, the contaminating bacterium will form a colony by growth and multiplication. Such a colony, though too small to be detected by the naked eye, will contain thousands of bacteria. During use, this colony will be spread all over the surface and thus form a very serious source of contamination, which may produce a wrong idea.

(2) Under no circumstances, should a plate be exposed to the open air, as bacteria may fall on the surface and contaminate it and make it useless.

(3) Inoculation or spreading of the material for examination may be done either by slightly raising the lid at one part to allow the introduction of the platinum loop or the spreader or *the plate may be held vertically with the left hand and inoculation done with the right* (Fig. 59). This will prevent micro-organisms from falling on the surface of the medium.

(4) A plate should be dry and must have no water of condensation on the surface. A moist plate when inoculated with a motile organism may give rise to a filmy layer of growth instead of discrete colonies. Before use, careful examination should be made for any moisture on the surface. If it is present, it should be allowed to evaporate by keeping the plate half open in the incubator for an hour or two.

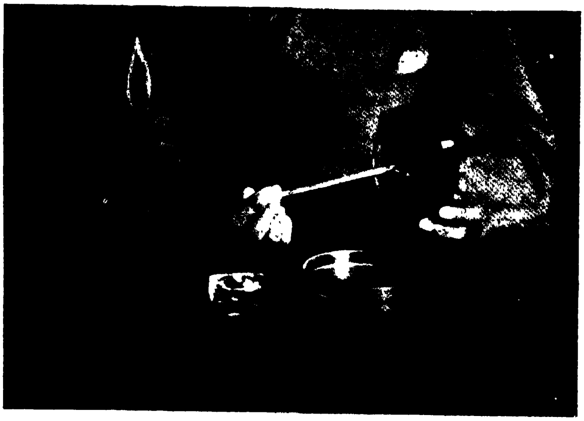


Fig. 59.
Method of holding a plate for surface culture with a spreader.

(5) After inoculation, all plates made up with agar, should be kept in the incubator in an inverted position, viz., with the lid down. This prevents any water of condensation which may collect on the lid from falling on the surface of the culture and thus producing a filmy growth. On the contrary, when gelatin plates are used, they should be kept exactly the opposite way, viz., the dish containing the medium should be below and the lid above. This prevents the liquefied medium from dropping on to the lid.

INCUBATION OF THE CULTURE

Most of the pathogenic bacteria grow best at blood heat, i.e., at a temperature of 37·5° C. The media after inoculation should therefore be exposed to this optimum temperature which must be maintained uniformly and continuously. This is effected in an apparatus known as the incubator (Fig. 60). The instrument consists of an inner water jacket and an outer case. The inner jacket is made of hard rolled copper and is a square box with double walls on five sides and on the sixth, a glass door. Thus the chamber is surrounded on all sides except the door by a water jacket which maintains an even temperature throughout all parts of the incubator. The interior of the chamber is provided with some movable shelves over which culture media are placed. The outer case is made of teak [illegible]d with asbestos with a door co[illegible]ding with the glass door. Th[illegible] between the inner jacket

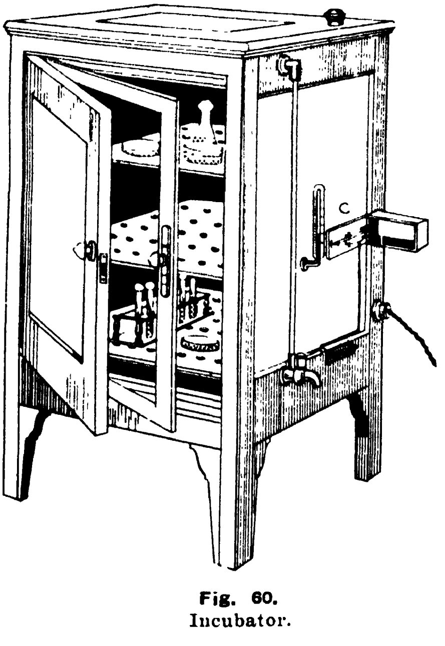


Fig. 60.
Incubator.

and the outer casing is heavily insulated with some non-conducting material. The inner jacket is filled with water which is then heated by electricity, gas or oil lamps. The temperature of the electrically heated incubators is regulated by an automatic capsule regulator (C). Once adjusted the temperature is automatically maintained within ½°C. Full directions for working the capsule are supplied with the incubator.

KEEPING OF STOCK CULTURE

Most of the bacilli can usually be kept alive by subculturing twice a month. During the intervals, the cultures are kept at 22°C., the cotton wool plugs being sealed with paraffin and covered with sterile rubber caps. The following are however exceptions to the general rule:—

Eberthella dysenteriæ, Shiga—to be subcultured every other day.

Eberthella paradysenteriæ, Flexner—to be subcultured twice a week.

Corynebacterium diphtheriæ—to be subcultured twice a week.

Hæmophilic group—every other day.

Klebsiella pneumoniæ—once a week.

As regards Cocci, Staphylococci and N. catarrhalis are to be subcultured twice a week; Streptococci, D. pneumoniæ, N. meningitidis and N. gonorrhœæ every day.

CHAPTER VII

STUDY OF CULTURAL CHARACTERS

The preliminary knowledge of the morphological and staining characters may, in certain circumstances, suffice to identify an organism but in general, study of the cultural characters is essential for its complete identification. Cultures are also necessary for studying the nature of toxin formation, serological reactions and pathogenicity tests. As a routine method, the organism is cultivated in nutrient agar, broth and blood agar media. Special media may be used for certain organisms or group of organisms which are unable to grow in ordinary media. This may be surmised, to some extent, from the clinical history of the case, source of the material and preliminary examinations.

Nutrient agar:—Colony-formation is studied in this medium. This is best examined after 24 hours' incubation at 37°C. The examination of the colonies is carried out with the naked eye, a hand lens, or the low power of a microscope, and attention is paid to the following:—

(i) Nature and distribution of colonies (discrete or overlapping), (ii) opacity (opaque, translucent or transparent), (iii) size, (iv) shape, (v) edge (even or irregular), (vi) surface (smooth or rough; raised, flat or depressed in the centre; dry or moist) and (vii) colour. Consistency of the colony is judged by touching it with a platinum wire and observing whether the colony is sticky and easily removed or remains adherent to the medium. The capacity to form a uniform emulsion in water or saline may also be noted.

After the colonies are examined as above, a film from each variety should be prepared and stained by the usual methods. If there are several types of colonies, subcultures should be made from each variety so that they are obtained in pure culture for further study.

Growth in fluid medium (Broth):—The attention is paid to the following:—(i) turbidity, (ii) nature of the deposit (flocculent or granular) and (iii) surface growth (pellicle formation).

Gelatin stab culture:—The form of growth (e.g. inverted fir-tree appearance of B. anthracis) and liquefaction of the medium are particularly observed.

Blood agar:—Certain bacteria, e.g., Str. hæmolyticus, when grown in this medium, produce a clear colourless zone round the colonies (Fig. 61). This is known as hæmolysis and is produced by the action of hæmolysins secreted by the bacteria concerned. It [illegible] seen later that such laking of blood may be produced by bacteria [illegible] not produce any hæmolysin; hence it is better to use the term hæm[illegible]ion instead of hæmolysis. A

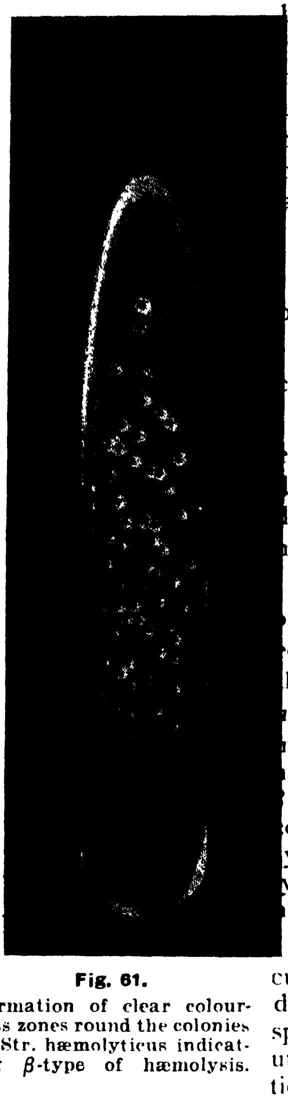


Fig. 61.
Formation of clear colourless zones round the colonies of Str. hæmolyticus indicating β-type of hæmolysis.

acterium may cause both hæmolysis and
æmodigestion. The formation of hæmolysin is
ore accurately demonstrated by adding red blood
ells to the filtrate of a broth culture. The factors
oncerned in the laking of blood and formation of
lear colourless zones around the bacterial colonies
re the following:—

(i) Disposal of hæmoglobin by the colony.

ii) Accumulation of hæmoglobin at the peri-
hery of the clear zone.

iii) Alteration of the hæmoglobin into a
olourless compound.

Some other bacteria,
.g., D. pneumoniæ, pro-
uce a green colouration
ue to the formation of
ethæmoglobin (Fig. 62).

The hæmolytic activity
f bacteria is well illustrat-
d by Streptococci and
hese can be classified into
, β and γ types, depend-
ng on the degree of their
æmolytic activity. Thus
-type means producing
omplete hæmolysis; α-
ype means partial hæmo-
ysis and γ-type means
o hæmolysis.

Apart from the above, cultural characters of different organisms in special media may also be utilised for the identification of bacteria. These special media contain substances which, while assisting the growth of the bacterium it is desired to cultivate, tend to restrain the growth of the associated organisms. They may also contain an indicator which changes colour when a particular organism develops in them. Thus, *the special medium may be either a selective medium or an indicator medium;* but for purpose of convenience, the two are often combined. ***Selective media*** may contain—

(a) Substances, such as blood, serum, glycerin, etc., which enrich the media and stimulate the growth of the organisms in question, e.g.

Fig. 62.
Green colouration round the colonies of D. pneumoniæ indicating the conversion of hæmaglobin into methæmoglobin (α-type of hæmolysis).

(i) Media containing hæmoglobin stimulate the growth of Hæmophilic group of organisms.

(ii) Media containing serum or egg stimulate the growth of Corynebacterium diphtheriæ.

(iii) Media containing ascitic fluid or hydrocele fluid stimulate the growth of Neisseria meningitidis.

(iv) Media containing glycerin stimulate the growth of Mycobacterium tuberculosis hominis.
And so on.

(b) Substances which, while favouring the growth of any particular organism, inhibit that of others, e.g.

(i) Bile salts in MacConkey's medium assist the growth of typhoid-dysenteriæ group of bacilli but inhibit that of cocci.

(ii) Telluric acid in tellurite medium assists the growth of diphtheria group but inhibits the growth of secondary organisms.

(iii) A highly alkaline medium, as Dieudonné's blood-alkali agar, inhibits the growth of ordinary intestinal bacteria but favours the growth of Vibrio comma (V. choleræ).

Indicator media:—The following are the examples of some indicators which are in common use.

Dye-stuffs:—These include litmus solution, neutral red, acid fuchsin, bromcresol purple and others. They are commonly used to indicate the production of acid from carbohydrates as in MacConkey's medium, litmus milk and various other sugar media. Thus, an organism growing in the MacConkey's medium may be a lactose-fermenter as indicated by the rose pink colonies (Colon group of bacillus) or a non-lactose-fermenter indicated by colourless colonies (Typhoid-dysenteriæ group).

Blood:—This is a useful indicator and is employed to note whether the organism in question is hæmolytic or non-hæmolytic. This is particularly used for the differentiation of streptococci.

Sodium tellurite:— Corynebacterium group of organisms reduces this substance which turns the colonies black.

Lead acetate:—This is used when the organisms produce H_2S; colonies are coloured black owing to the production of lead sulphide and this indicator is particularly used in the differentiation of Salmonella paratyphi (B. paratyphosus A) and Salmonella schottmülleri (B. paratyphosus B); the latter reduces the lead acetate.

It has already been mentioned that before making cultures, a stained film of the material should always be examined to get an idea about the nature of the infective organism and the media in which inoculation is to be made. In this way, the special media that may be required for a particular organism may be surmised to a certain extent. For common laboratory work the following system is usually fol[illegible]

Inoculating material.	*Media to be inoculated.*
Sputum	Nutrient agar, blood agar and broth.
Throat swab	Nutrient agar, blood agar and broth. If there is a suspicion of diphtheria, egg medium, Loeffler's serum medium or serum agar medium may be employed.
3. Fæces	MacConkey's medium (plate), bromcresol purple medium (plate) and broth.
4. Urine	MacConkey's medium (plate) and bromcresol purple medium (plate).
5. Pus	Nutrient agar, blood agar and broth.
6. Blood	Glucose broth or bile broth.
7. Cerebrospinal fluid	Nutrient agar, blood agar and hydrocele fluid agar.
8. Urethral smear	Same as Cerebrospinal fluid.
9. Uterine swab	Nutrient agar (plate), blood agar (plate) and broth.
10. Conjunctival swab	Nutrient agar and blood agar.

Note:—For the study of colonial characters, it is advisable to have the media in Petri dishes.

Although the cultural characters of different groups of bacteria are constant, yet such a study merely indicates the group to which a particular organism belongs. It does not differentiate between the individual members of the group, which are similar in their morphological and cultural characters. For further differentiation, a detailed study should be made regarding their metabolic and biochemical properties, serological reactions and pathogenicity. Thus the study of cultural characters serves only to confirm the inference deduced from the study of morphology and staining reactions.

Lastly, it should be remembered that besides artificial media, *animal inoculation method* may also be employed for the cultivation of some organisms. In this method, the infective material is inoculated into a susceptible laboratory animal and the organism is recovered in pure culture either from heart's blood, spleen or from the tissues in which the lesions are produced. These laboratory animals represent, as it were, the living culture media. The important organisms which may be isolated by this method are Diplococcus pneumoniæ and Mycobacterium tuberculosis.

STUDY OF BIOCHEMICAL REACTIONS

These are of immense value in the differentiation of bacterial species. They are so very important that they afford a delicate method of finding out even the minuter differences present in the closely related bacteria. Under this heading the following are studied.

(i) Fermentation of "Sugars."
(ii) Coagulation of milk.
(iii) Proteolytic powers.
(iv) Production of indol and nitroso-body from peptone.
(v) Power of reduction.
(vi) Formation of H_2S.
(vii) Methyl-red tests.
(viii) Voges-Proskauer reaction.
(ix) Utilisation of citrates (Koser's test) and uric acid.

(i) ***Fermentation of "Sugars":***—Media used for the purpose are (a) fluid media which include (1) broth (sugar free) or peptone carbohydrate medium or (2) Hiss's serum water medium and (b) Solid media.

Peptone carbohydrate media (1 per cent. peptone water containing 1 to 2 per cent. sugar):—When an organism is cultivated in such a medium, it may either fail to produce any change or may ferment the carbohydrate producing acid only or an acid reaction accompanied by evolution of gas.

To detect the formation of acid, an indicator is included in the medium. The liberation of gas is rendered evident by an inverted Durham's tube which is also incorporated in the medium. The gas evolved is generally a mixture of hydrogen and carbon dioxide and collects as bubbles at the top of the Durham's tube. The proportion of two gases is sometimes important, particularly in the differentiation of saprophytic and fæcal types of Escherichia coli. The sugars commonly employed are lactose and glucose but in special work, other carbohydrates may also be used.

Hiss's serum water medium:—This medium is used for certain organisms, such as D. pneumoniæ, C. diphtheriæ, etc., which grow poorly in broth or peptone carbohydrate media. It contains serum, an indicator (litmus solution) and fermentable substances; it does not contain any inner tube. A bacterium grown in this medium may ferment the carbohydrate producing acid which is shown by the indicator and also by the coagulation of the medium.

Solid media:—When the amount of gas is small, the fermentative capacity of bacteria may be judged by using a solid medium (agar) containing fermentable carbohydrates and an indicator. The tubes are inoculated in the melted condition and then allowed to se [illegible] ter incubation, acid production is recognised by a change in the colour o [illegible] dicator and gas produced is judged by the splitting up of the medium [illegible] he collection of bubbles in it.

(ii) ***Coagulation of milk:***—The medium used for the purpose is litmus milk. The coagulation is produced by a ferment coagulase. Carbohydrate fermentation (lactose) and proteolytic digestion may also be studied in this medium.

(iii) ***Proteolytic power:***—Digestion of egg, serum or gelatin is observed in the respective media.

(iv) ***Formation of indol and nitroso-body:***—The organism is grown in broth or peptone water and the production of indol and nitroso-body is detected by the following methods:—

Nitroso-indol method:—When the organism produces both indol and nitrite, the addition of a few drops of sulphuric acid will produce a rose colour indicating their presence. Such a reaction is obtained with cholera vibrio and is known as "Cholera red reaction." When the organism, as in the case of the Colon group, produces indol only, the rose colour is obtained by adding a few drops of dilute solution of nitrate (0·1 per cent. potassium nitrate) and a few drops of sulphuric acid.

Ehrlich's rosindol reaction:—Into a test tube containing about 2 to 3 c.c. of peptone water culture, the following reagent is added. A rose colour develops in the presence of indol.

Reagent:—

Para-dimethyl-amido-benzaldehyde ...	1 gm.
Absolute alcohol ...	95 c.c.
Concentrated hydrochloric acid ...	20 c.c.

The addition of a saturated solution of potassium persulphate hastens the process.

(v) ***Power of reduction:***—The reduction of telluric acid is observed by the formation of black colonies in the tellurite medium and is specially used for the differentiation of Corynebacterium group of organisms.

Reduction of nitrate:—Certain organisms, such as Escherichia coli, are capable of reducing nitrate into nitrite. The organism is allowed to grow in a fluid medium, such as broth or peptone water to which 0·1 per cent. potassium nitrate has been added. After 2 to 3 days of incubation at 37°C., the formation of nitrite is detected by Ilosvay's method. Two solutions are required for the purpose:—

Solution A:—This is prepared by dissolving 1 gm. of *a*-naphthylamine in 22 c.c. distilled water. It is then filtered and to the filtrate 180 c.c. of dilute acetic acid of 1·04 specific gravity are added.

Solution B:—This is prepared by dissolving 0·5 gm. of sulphanilic acid in 150 c.c. of dilute acetic acid. In carrying out the test 1 c.c. of each of the above solutions is added to the broth culture and the resulting colour noted. The positive reaction is indicated by the immediate development of a red, rose-pink or maroon colour whereas the negative reaction by no colouration.

(vi) ***Formation of hydrogen sulphide:***—This is tested on lead acetate agar. The appearance of a brown or black colour shows a positive result. This test is utilised in the differentiation of Paratyphoid group of organisms.

(vii) ***Voges and Proskauer reaction:***—This reaction depends on the production of acetyl-methyl-carbinol from glucose by certain organisms when grown in a buffered glucose broth (p. 73). The test is carried out by growing the organism in such a medium for 3 or 4 days and then adding 1 c.c. of a 10 per cent. solution of KOH. The mixture is then shaken vigorously. A positive reaction is indicated by the appearance of a pink fluorescence, a colour resembling that of a weak solution of eosin. It is better to read the test after 18 to 24 hours, as it may require several hours before the colour is well-developed. A negative reaction is indicated by no colouration.

(viii) ***Methyl-red test:***—The test depends on the ultimate reaction that is attained after fermentation of glucose in the same medium as that utilised for Voges and Proskauer test. After 4 days' incubation at 37°C., five drops of 0·04 per cent. solution of methyl-red (0·1 gm. of methyl-red dissolved in 300 c.c. alcohol and made up to 400 c.c. with distilled water) are added as an indicator and the resulting colour noted. Appearance of a red colour signifies positive and a yellow colour a negative reaction. The former indicates persistent acidity and the latter indicates that primary acidity has been replaced by alkalinity. This test along with V. P. test is employed for classifying the coliform bacilli isolated from water supplies.

(ix) ***Utilisation of citrates and uric acid:***—The former is tested in *Koser's medium* (p. 74). The bacteria which are not able to utilise the citrates will not grow in this medium. This is particularly used in the differentiation of faecal and saprophytic types of Escherichia coli. Uric acid medium (p. 74) may be employed for the same purpose.

CHAPTER VIII

PATHOGENICITY TESTS

The crucial test for the identification of an organism is the pathogenicity test which depends on its ability to produce disease in various susceptible animals. For this purpose, inoculation experiments are performed on the common laboratory animals. This method takes advantage of the fact that some animals are very susceptible to certain bacteria which produce lesions so characteristic as to be considered specific for that particular bacterial species. A study of such lesions at autopsy will enable one to establish its identity definitely. This is true of Mycobacterium tuberculosis, Corynebacterium diphtheriæ, B. anthracis, D. pneumoniæ and others. Besides the pathogenicity test, such inoculation experiments may also be performed for other purposes, e.g.

(i) To isolate the organism in pure culture (Diplococcus pneumoniæ, Mycobacterium tuberculosis, etc.).

(ii) To increase the virulence (exaltation) of an organism, e.g., exaltation of the virus of hydrophobia by passage through rabbits.

(iii) To decrease the virulence (attenuation) of an organism, e.g., attenuation of the virus of smallpox by passage through calves.

(iv) To prepare antisera which may be antitoxic or antibacterial.

(v) To study the neutralisation of antigen by its specific antibodies.

Animals:—The common laboratory animals used for the purpose are guinea-pigs, rabbits, rats and mice. Monkeys are also being used. The ideal animal for this sort of experiments would be man but it is forbidden under the laws of the civilised world, although instances are on record where condemned criminals and volunteers have been used for such purposes. Owing to this reason, we are content with lower animals or animals very near to man in the scale of creation.

Guinea-pigs are the common laboratory animals and are used in the diagnosis of the following diseases:—tuberculosis, diphtheria, anthrax, plague, glanders, spirochætal jaundice, cholera, gas gangrene and botulism.

Rabbits are very prone to parasitic and intercurrent infections, hence they are less satisfactory animals than guinea-pigs for experimental purposes. They are used particularly in the diagnosis of rabies (hydrophobia) by subdural inoculation of an emulsion of brain tissue from a suspected dog. In tuberculosis, rabbit is mainly used to differentiate M. tuberculosis bovis from M. tuberculosis hominis. In the laboratory, rabbits are also used for the preparation of agglutinating and hæmolytic sera.

Rats are used for the same purpose as guinea-pigs.

Mice are largely employed for the isolation of Diplococcus pneumoniæ (Pneumococcus) in pure culture and are now extensively used for determining the various types of this organism.

Monkeys are mostly used for investigating the mode of infection and pathogenicity of many respiratory diseases and virus infections, such as acute anterior poliomyelitis and encephalitis lethargica.

Methods of inoculation

1. **Skin scarification:**—A careful epilation is an essential preliminary. Several scratches are then made on the belly which may be either parallel or at right angles to the longitudinal axis of the body. The infective material is then gently rubbed in with a sterile glass rod or spatula over the scarified area. This method is used to determine the route of general infection, e.g., the transmission of plague in rats.

2. **Intradermal inoculation:**—This is chiefly used to test local inflammatory skin reactions, e.g., in the standardisation of the toxins of Corynebacterium diphtheriæ. Preliminary epilation is also necessary for this purpose. The animals used are rabbits and guinea-pigs.

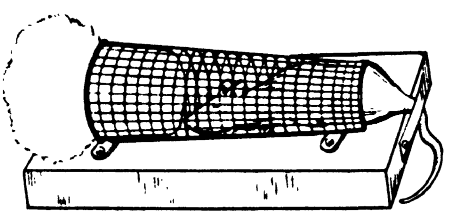


Fig. 63.

Method of holding the rat for intravenous inoculation into the dorsal root vein of the tail.

3. **Subcutaneous and intramuscular injections:**—These are the usual methods employed and present no difficulty.

4. **Intravenous injection:**—In mice, one of the tail veins is utilised. The animal is put in a special cage as shown in Figure 63 and the veins are dilated by immersing the tail in warm water. The vein at the root of the tail is then selected. In guinea-pigs and rabbits, one of the marginal veins in the ear is selected; the veins are made prominent by rubbing the area with ether or xylol. Figure 64 shows the method of holding the animal for intravenous injection.

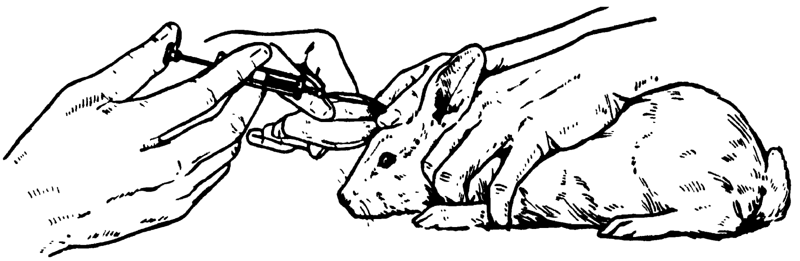


Fig. 64.

Method of holding the rabbit for intravenous inoculation into the marginal ear vein which has been made prominent by the assistant holding it between the thumb and the [illegible] fingers.

5. **Injection into special regions** [illegible] as intrathecal, intracerebral, intratesticular and intratracheal is also [illegible]d.

6. **Inhalation:**—By this method the material is sprayed into the nose or the animal is allowed to inhale the infected material. This is employed in the experimental inoculation of tuberculosis in guinea-pigs.

7. **Ingestion:**—By this method, the infective agent is either mixed with food material which the animal is made to eat or introduced into the stomach through a tube. Experimental tuberculosis in guinea-pigs may also be carried out by this method.

The methods will however vary according to the disease or the infecting organism for which they are used, as shown in the table:—

Disease and the infecting organism.	*Susceptible animal.*	*Methods of inoculation.*
Diphtheria (C. diphtheriae), tuberculosis (M. tuberculosis), anthrax (B. anthracis) and botulism (Cl. botulinum).	Mice or guinea-pigs	Subcutaneous
	Guinea-pigs	
Plague (P. pestis)	Guinea-pigs or Rats	Subcutaneous or intracutaneous.
	Guinea-pigs	Intraperitoneal
Pneumococcal and streptococcal infections.	Mice	Subcutaneous or intraperitoneal.
	Rabbits	
Rabies (Virus (?) infection)	Rabbits	Subdural
Gas gangrene (Cl. welchii & others)	Rabbits	

Nature of the inoculated material

(i) **Fluid exudates,** such as pus; cerebrospinal, pleural, peritoneal, synovial and pericardial fluids; blood; and urine.

(ii) **Cultures:**—Fluid cultures are directly inoculated. Cultures on solid media are first emulsified in saline and then injected.

(iii) **Toxins (exotoxins):**—A bacteria-free filtrate is obtained by filtering a bouillon culture through a porcelain filter.

(iv) **Tissues:**—These should be cut into pieces with sterile scissors and ground up in a mortar with sterile sand. After completion of the process, the contents are shaken with saline in a test tube and then allowed to stand. The sand and solid particles settle at the bottom and the supernatant fluid taken out for injection. For intravenous injections all solid particles are removed by centrifugalising at a low speed.

Observation after inoculation:—The animals, after inoculation, are kept in cages made of galvanised iron wire. The general condition of the animal is observed and special attention is paid to the following:—attitude, appetite, weight and temperature. Where it survives a longer period, the weight of the animal is frequently a guide to what is happening. Hence inoculated animals should be weighed at regular intervals. Rapid loss of weight indicates a serious infection or toxæmia. The temperature of the animal is usually taken per rectum.

How to kill the animals:—A general anæsthetic, such as chloroform, is usually employed for the purpose but it causes much struggling, resulting in petechial hæmorrhages in the lungs and central nervous system, which may obscure the finer histological details in those organs. One of the most satisfactory methods is to poison the animal with coal gas and its use has a further advantage of economy. The gas should be administered slowly. The animal may also be killed by injecting 10 per cent. chloretone in olive oil either subcutaneously or intraperitoneally.

Autopsy

It is desirable that the post mortem examination should be performed as soon as the animal is dead or killed. In cases of delay, it may be kept in the cold chamber.

Apparatus required:—Cork or wooden boards or paraffin trays; pins, trays, and surgical instruments, such as scalpels, dissecting forceps and scissors. Bacteriological requisites include sterile swabs, sterile capillary pipettes and rubber teats, sterile Petri dishes, sterile test tubes, platinum loops, glass slides and coverslips, fixing fluids, culture media, spatula for searing and spirit lamp.

Examination:—For a bacteriological post mortem examination, an aseptic technique is essential and the worker should protect himself with rubber gloves. Larger animals, such as rabbits and guinea-pigs, are nailed out on a wooden board and smaller animals such as mice are best pinned out on a thick cork. The examination is then conducted in a routine and orderly manner.

Final disposal of carcasses:—As these animals may contain virulent organisms, dangerous to human life, all precautions should be taken to prevent dissemination of the disease. For this reason, the animal, after the examination is over, may be dipped into a [illegible] antiseptic lotion, e.g. lysol, phenol or formalin, but the carcasses should [illegible] be burnt in an incinerator.

Identification of organisms by Pathogenicity tests: Common examples:—

Organism.	*Susceptible animal used.*	*Characteristic reaction and lesions produced.*
1. C. diphtheriæ	Guinea-pigs	Animals die in two to four days. Post mortem, adrenals are found to be enlarged, swollen and hæmorrhagic.
2. M. tuberculosis	Guinea-pigs	Death in 6 to 12 weeks. Post mortem, local caseous abscess with numerous tubercle bacilli; spleen, liver, lungs and lymph glands are enlarged and studded with yellowish or greyish white areas (caseation) of necrosis.
3. B. anthracis	Mice, guinea-pigs and rabbits	Animals die in 12 to 48 hours. Post mortem, hæmorrhagic local exudate, enlarged spleen and bacilli in blood and organs.
4. D. pneumoniæ	Mice	Animals die in about 2 days and the blood teems with D. pneumoniæ.
5. P. pestis	Guinea-pigs, mice and rats	Animals die within 2 to 7 days. Post mortem, hæmorrhagic necrosis at the site of injection with the involvement of the proximal lymph gland. Spleen, liver, lungs and other lymph glands are studded with greyish white nodules and hæmorrhagic spots. Organisms are found in large numbers in the local lesion, spleen and heart's blood.
6. Cl. welchii and other organisms of gas gangrene	Rabbits intravenously	Animals are killed after a few minutes and then placed in an incubator for 5 to 6 hours. The animals will be found to be distended with gas and particularly the liver is found to be riddled with gas bubbles (foamy liver). The organisms may be isolated from the liver and heart's blood.

VIRULENCE OF BACTERIA

***Definition:*—**Virulence of a bacterium may be defined as its ability to invade and multiply in the tissues of a susceptible host. In other words, it is the power of pathogenic micro-organisms to incite disease in the animal or man.

Attenuation and exaltation of virulence:—Virulence of a bacterium may be increased or decreased by various means. The process by which the virulence is diminished is called *attenuation* and that by which it is increased is called *exaltation.*

Attenuation:—This indicates diminution of the virulence of an organism and is, generally speaking, induced by repeated subcultures in artificial media. The following are the various methods by which attenuation of a virus is brought about:—

(1) *By passing the organism through the body of a less susceptible animal,* e.g.,

(a) Virulence of anthrax bacilli for bovine animals is attenuated by passing the organism through some resistant animals, such as guinea-pigs and rats. The subsequent inoculation into cattle of B. anthracis from guinea-pigs, produces a disease which is non-fatal.

(b) In the same way, the virulence of smallpox virus is diminished by passage through calves.

(2) *By repeated subcultures in artificial media outside the animal body,* as in Streptococci, D. pneumoniæ and E. typhi.

(3) *By growing the organisms in unfavourable conditions*—When grown at an abnormally high temperature or in strong light, the virulence of some bacteria is diminished, e.g., attenuation of B. anthracis is carried out by growing the organism at a temperature of 42°C. and vaccines of such a strain are used for prophylactic inoculation against sheep and cattle.

(4) *By growing organisms in the presence of weak antiseptics,* e.g., virulence of bovine tubercle bacillus is attenuated by culturing in the presence of bile. This is used in the preparation B. C. G. vaccines.

(5) *By desiccation,* e.g., attenuation of Hydrophobia virus is carried out by drying the spinal cords in the air. The degree of attenuation being proportional to the time of exposure.

(6) *By prolonged keeping*—All bacteria on keeping (i.e., as they grow old) tend to lose their virulence.

Exaltation of virulence means increase of virulence. This is accomplished by quick passage through susceptible animals by successive inoculations. A classical example is Hydrophobia virus. This was first done by Pasteur by repeated subdural inoculation into rabbits, the virus being thus exalted and its virulence increased. V. [illegible], E. typhi, D. pneumoniæ, Streptococci all increase in virulence [illegible] passed through susceptible animals.

In connection with the virulence of a bacterium, it should be remembered that at the beginning of an epidemic disease, when the bacterial strain is comparatively young, its virulence is exalted to a very considerable extent, owing to rapid passage from one human being to another who have had no time to develop immunity. This explains the virulence of an epidemic and its high rate of mortality at the beginning. Then again, towards the end of the epidemic, when the causative organisms grow older and also when there is development of a certain amount of mass-immunity, the virulence of bacteria is attenuated by its passage through immune human beings. This explains the gradual decline of an epidemic with its fall in mortality.

Relation of aggressins to virulence (Bail's theory):—Pathogenic bacteria manage to overcome the protective mechanisms of the animal body and to proliferate within it by virtue of a definite substance given off by them (aggressins), which acts primarily by protecting the bacteria against phagocytosis and by repelling leucocytes. It has already been mentioned that aggressins are substances produced by bacteria during their growth in the animal body.

Relation of capsules to virulence:—The bacterial capsules seem to insulate them against the attacks of the body cells and fluids and hence the organism can multiply freely in the tissue producing harmful effects. Capsules, as mentioned before, reach their fullest development in the animal body, but the bacteria tend to lose them in an artificial culture. A virulent strain of D. pneumoniæ, when it loses its capsule, becomes avirulent. Thus the presence of a capsular substance is distinctly associated with the virulence of an organism.

Recent researches on the relation of virulence to the characters of the growth

Avirulent variant:—If an organism, say E. typhi, is grown repeatedly, in artificial culture media, after sometime, two distinct races of organisms, as seen by differences in the character of the colonies, are developed. One of these races produces rough, granular colonies on the surface of agar and a floccular growth in broth tending to sedimentation. The other race produces usual smooth colonies on agar and uniform turbidity in broth. The organisms composing the rough colonies are found to become less virulent and cease to agglutinate in type sera. In cases of capsulated bacteria, such as D. pneumoniæ, transformation from the normal smooth to the rough colonies, results in the disappearance of the capsule and hence the organism in the rough stage becomes avirulent.

CHAPTER IX

INFECTION

DEFINITION:—When micro-organisms invade the body of animals or human beings and give rise to disease, the process is known as infection. It should be definitely understood that the mere presence of micro-organisms in the body of an animal does not constitute infection. Bacteria are present almost everywhere in the environment of animals and human beings and some of them find constant lodgments on various parts of the body. For instance, pathogenic bacteria may be present in the mouth, skin, digestive tracts and various other places in our body without causing infection. For the latter purpose, however, there must be growth of those bacteria, with diffusion of their poisonous products and a consequent tissue reaction. The bacteria are divided in this respect into two great classes:—

1. ***Saprophytes:***—Those living on dead organic matter.

2. ***Parasites:***—Those living on and in, the animal body and may be either commensals or pathogens.

(a) **Commensals:**—There are numerous species of bacteria which normally inhabit various parts of the body, such as skin, conjunctiva, mouth, nasal passages, upper respiratory tracts, intestines and gential ducts (vagina). [Vide normal bacterial flora of the body]. Under normal circumstances, most of them do not produce any definite disease, but when they depart from their normal habitat and invade the tissues of the body, they initiate disease process. Thus, so long as Escherichia coli remains in the intestines, it is harmless, but its invasion into the body may give rise to cystitis, pyelitis or abscess formation. Similarly, streptococci which are constantly present in the mouth as harmless commensals may cause subacute endocarditis, arthritis, etc., when they gain entrance into the body. Thus the commensals may be (i) *NON-PATHOGENS* or (ii) *FACULTATIVE PATHOGENS*, i.e., given an opportunity, they will prove pathogenic. Hence the latter are called *opportunists* by Theobald Smith.

(b) **Pathogens:**—The chief function of these bacteria is to produce disease in man and animals. They are not normally present, but gain entrance into the body from another source and reach the system through certain avenues.

Before such organisms are definitely incriminated as the causative agents of a disease, they must fulfil certain conditions which are said to have been summarised by Koch and handed down to students of bacteriology as ***Koch's postulates.*** These are as follows:—

(1) The particular organism must be constantly found in the lesions and present in the animal either dead or alive

(2) They must always be cultivated in [illegible]ial culture media and isolated in pure culture from those pathogenic le[illegible].

(3) When inoculated into susceptible animals, they should be capable of reproducing the disease.

(4) From the inoculated animal and its lesions, the same micro-organism must be recovered in pure culture.

Although such postulates were thought to be very good practical rules to establish the identity of a particular organism with any particular disease process, they are now found to be incomplete and even defective in many ways. For instance, the demonstration of the development of antibodies in the blood serum of an infected animal forms an important diagnostic criterion, even if the infective agent cannot be isolated in pure culture. Again, there are some diseases where, although the causative organisms are definitely and constantly found in the lesions, they cannot be cultivated outside the host and isolated in pure culture. The commonest example of such a disease is leprosy in which the Mycobacterium lepræ are constantly present in the lesions, but they have not been cultivated and isolated in pure growth outside the tissues of the human host. Again, with the rapid advance of science, many diseases have been found to be caused by some sort of organisms which, being too small to be seen with the highest power of the modern microscopes, are not seen in the lesions, nor can they be cultivated and isolated as demanded by the Koch's postulates. Such are the many *virus diseases.* The question of the reproduction of the disease by inoculation of the infecting germ is also to be modified. For example, nobody has ever been able to reproduce leprosy in the animal and still the Mycobacterium lepræ is the cause of the malady. Besides, many of the diseases such as typhoid fever, cholera, diphtheria, scarlet fever, etc., are not exactly reproduced in the animal in the same form as they are found in man and as required by the Koch's postulates. Thus, it will be seen that the above maxims cannot be accepted *in toto* and the time has come for modifying them so as to meet the modern advances in bacteriology.

It is now necessary to consider how the infecting organisms are spread from one victim to another and the route by which they invade the body.

SOURCES OF INFECTIONS:—The diseases which are communicated to man are obtained chiefly from two sources, viz. (1) *man himself* and (2) *the lower animals.* Man is the great source and reservoir of most human infections. Man is the man's greatest enemy, as most of the diseases which are specific for human beings are communicated from man to man. The following are some of the diseases which are transferred from lower animals to man. (Fig. 65):—

Glanders from horse.
Tetanus from horse, cattle and sheep.
Tuberculosis (partly) from cattle.
Anthrax from cattle and sheep.
Malta fever from goat.
Rabies from dog.
Rat-bite fever and Weil's disease from rat.
Plague from rat (Rat flea being intermediary).

SOURCES OF INFECTIONS

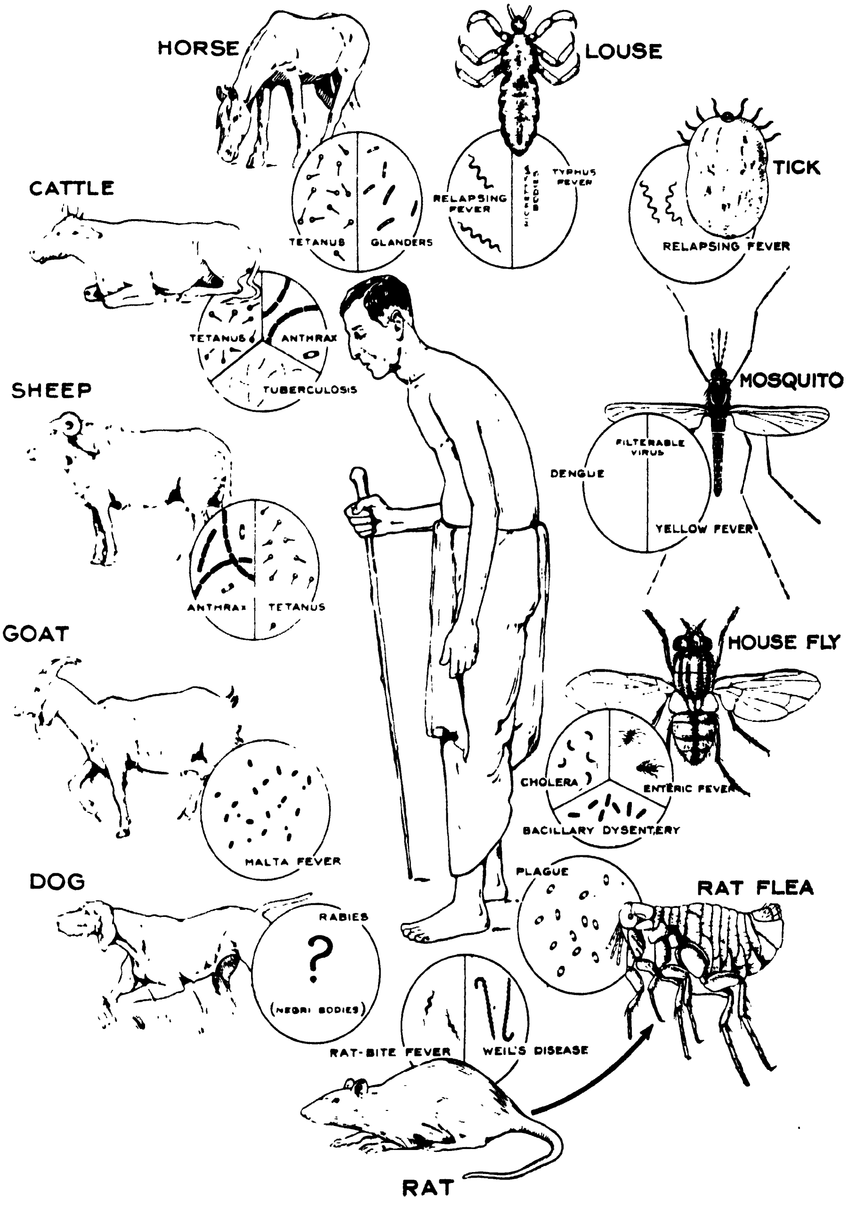


Fig. 65.

Diagram illustrating the sources of infection of some [illegible] the common *bacterial* diseases. The names of diseases and the organisms concerned are inserted within the circle. Rôle of rat in the transmission of plague is shown [illegible] arrow. The sign ? indicates that the nature of the organism [illegible] unknown.

MODES OF TRANSFERENCE:—*These may be described under three* heads:—(1) direct, (2) indirect and (3) through intermediate hosts.

1. ***Direct:***—Bacteria may be transferred more or less directly from person to person by what is known as *contact infection*. The contact may be with actual cases or with carriers. In this mode of infection, the organism may be transferred from man to man directly by intimate contact. Among the diseases in which contact is usually direct, the outstanding examples are gonorrhœa and syphilis and they are correctly called contagious. But in other cases, actual contact between the two individuals may not be necessary, the transference being effected through air by what is known as *droplet infection*. In this method, the causal organisms leave the body through discharges (saliva and sputum) from the mouth and nose and are sprayed out in the air during coughing, sneezing and speaking. They are then inhaled by a fresh victim who happens to come in contact with these persons. It is for this reason that overcrowding and lack of ventilation render transfer in this way particularly easy. Droplet infection is responsible for many epidemic outbreaks and always plays a dominant rôle in the spread of many infectious diseases, such as tuberculosis, pneumonia, common cold, cerebro-spinal fever, diphtheria, whooping cough, measles, mumps, etc.

2. ***Indirect:***—By this method, the organisms are transferred indirectly from person to person through water, food, soil and *fomites*. In a large majority of the diseases contracted by indirect infection, the organism is taken into the system through the mouth. The common examples of this class of diseases are typhoid fever, cholera and dysentery. In this connection it should be remembered that common house-flies play a very important part in the dissemination of the above diseases. They act as mechanical carriers by transferring the infecting agent from contaminated excreta and discharges to food. Other examples of indirect contact causing disease, are the sucking of a pencil alternately by the diphtheria-carrier and his school-fellows, thereby disseminating diphtheria. Similarly, the midwife's or nurse's or doctor's hands may convey the organism of puerperal sepsis from case to case.

3. ***Through an intermediate host:***—In certain diseases, a blood-sucking insect transfers the organism from individual to individual (Vide Fig. 65), e.g., plague by rat-fleas, yellow fever and dengue by mosquitoes (Stegomyia) and relapsing fever by ticks and lice.

CHANNELS OF INFECTION:—The micro-organisms causing disease may enter the body by the following channels:—

1. Respiratory tracts, e.g., the diseases which are spread by droplet infections, as in the case of pulmonary tuberculosis.

2. Digestive tracts, e.g., the diseases which are disseminated by contaminated food and drink, as in cholera, typhoid fever and dysentery.

3. Genito-urinary tracts, e.g., the diseases which are contracted by sexual intercourse, as in syphilis, gonorrhœa and tuberculosis (rarely).

4. Skin, e.g., the diseases which are transmitted by blood-sucking insects (Insect-borne diseases).

Note:—(i) About 90 per cent of all infections are taken into the body through the mouth.

(ii) Organisms taken in by the mouth and nose, do not necessarily cause respiratory infections. As for example, in cerebrospinal fever, meninges of the brain and the spinal cord are involved and in acute anterior polimyelitis, anterior horn cells of the spinal cord are involved, although in both cases, the portal of entry is the upper respiratory tract.

CERTAIN TERMS IN CONNECTION WITH INFECTION AND INFECTIOUS DISEASES

Fomites:—The term usually refers to inanimate objects such as beddings, clothings, drinking cups, pencils, toys and other objects, which are capable of absorbing, retaining and transporting infectious agents.

Carrier: A person who harbours pathogenic micro-organisms in his body, but who, nevertheless, shows no signs or symptoms of the disease, is known as a *carrier*. It has been definitely found that during an epidemic outbreak, many more persons carry the germs of the disease than those who actually contract the illness. Although the carriers themselves escape from an acute attack, they always serve to scatter the germs and spread the disease.

Contagious and infectious:—A *contagious disease* implies direct or personal contact, the best example of which is syphilis and gonorrhœa. An *infectious disease* may not be conveyed directly and so obviously as the former, but indirectly, through some hidden influence or medium. *Thus, infectious diseases may be contagious and contagious diseases are infectious.*

Epidemic, Endemic, Pandemic and Prosodemic

Epidemic:—A disease is said to be epidemic, when it spreads rapidly and attacks many people at the same time. (A similar condition amongst animals is called *epizootic*).

Endemic:—A disease is said to be endemic, when it is constantly present, to a greater or less degree, in any particular place. Insect-borne diseases are best examples of endemicity. "*An endemic disease smoulders, whereas an epidemic bursts into flames.*"

Sporadic:—A disease is said to be sporadic, when a few scattered cases occur now and then.

Pandemic:—A disease is said to be pandemic, when a large number of people in many countries are affected at the same time, i.e., a disease which is more or less epidemic everywhere. As for example, influenza was pandemic on two occasions (1889-90 and 1918-19); plague was pandemic on many occasions.

Prosodemic:—This term refers to those endemic diseases which are creeping or smouldering and are transmitted from person to person chiefly by contact. The best example is Cerebrospinal fever.

FACTORS WHICH PREDISPOSE TO BACTERIAL INFECTION

In order that infection may take place, certain essential conditions must be fulfilled and this may be considered under two heads: (A) those which are concerned with the infecting agent and (B) those which are concerned with the subject of infection.

A. INFECTING AGENT:—In the case of a particular bacterium, its effect will depend chiefly upon the following:—

1. *Virulence of the organism.*
2. *Number of the organisms introduced, i.e., the dose of the infecting agent.*
3. *Appropriate avenue of infection.*
4. *Defence of bacteria, i.e., nature of the resisting powers of the bacterium.*

1. ***Virulence:***—Virulence of a bacterium determines, other things being equal, whether or not infection will occur. The pathogenicity of a bacterium depends on the invasiveness and toxigenic properties of the organism concerned. By invasiveness we mean that the organism has greater capacity for multiplication in tissues, or are more able to resist phagocytosis and other destructive agencies. This is best exemplified by the haemolytic strain of streptococci, which produces a fatal infection owing to its invasive property, in addition to toxin production. Again, there are species of bacteria which do not spread widely in tissues, but their degree of virulence depends entirely on their power of toxin production. Corynebacterium diphtheriæ and Clostridium tetani are strong toxin producers with but little invasiveness.

2. ***Number of the organisms (Dose of the infecting agent):***—Healthy tissues can usually resist a certain number of pathogenic organisms of a given virulence. A small number, received intermittently, will probably be destroyed and this may lead to an increased immunity for that particular organism. When, however, the organisms are received in massive doses and in quick succession, infection is most likely to occur (*Cf.* Tuberculosis). The current dose of infection depends on the proximity of the source and the time of exposure, and these again are largely influenced by overcrowding and lack of ventilation. Hence, these factors are to be seriously considered in the prevention of many infectious diseases (particularly respiratory diseases).

3. ***Avenue of infection:***—The bacteria must gain entrance into the body by a path best adapted to their own requirements which shall permit them to proliferate, after gaining a foothold. Certain organisms, such as Vibrio comma, Eberthella typhi and Eberthella dysenteriæ, will not produce any intestinal disease when injected subcutaneously. For the production of specific diseases, they must gain entrance through the alimentary tract. Similarly, Neisseria gonorrhœæ can produce disease, if they enter the body through the mucous membrane of the genitalia or the conjunctiva.

4. ***Defence of bacteria:***—Bacteria are provided with certain equipments which help them in overcoming the defensive mechanisms of their hosts and these are as follows:—

(i) **Presence of capsules:**—Some bacteria insulate themselves in the animal body by the formation of capsules. It has been mentioned before, that there is some relation between capsule formation and virulence of a bacterium. This is particularly seen with Diplococcus pneumoniæ (Pneumococcus); the capsulated form is distinctly virulent, whereas the non-capsulated form is avirulent.

(ii) **Spore-formation:**—Certain other bacteria have the property of developing spores, when placed under adverse circumstances; when this is overcome, i.e., in favourable conditions, they again develop into vegetative forms and are thus capable of producing disease.

(iii) **Products of bacterial growth:—**

(a) *LEUCOCIDIN:*—This is a substance produced by certain bacteria, particularly staphylococci, which has the property of destroying leucocytes.

(b) *AGGRESSINS:*—These substances are secreted by the living uninjured bacteria. They are not derived by solution of bacteria. It has been claimed by *Bail* that production of aggressin is definitely related to virulence of bacteria. Aggressin helps infection by inhibiting the defensive mechanism, particularly, if not solely, by blocking phagocytosis, as well as repelling the leucocytes (negative chemiotaxis). There are certain bacteria which are notable for the production of aggressin, the most important being the anaerobic organisms of gas gangrene.

(iv) **Development of resistant strains:**—This is particularly met with in the spirochætes of relapsing fever. After the crisis, a few spirochætes which are not destroyed by the immune bodies of the serum survive and develop into resistant forms. These are serologically a distinct strain and are responsible for the occurrence of a series of relapses from which the patient suffers, and for which the disease is called relapsing fever.

B. SUBJECT OF INFECTION:—For infection to take place, susceptibility of the host is also important. Susceptibility is the reverse of immunity; when one is high, the other is low. Susceptibility to a particular micro-organism varies according to species and racial peculiarities. These questions are considered in greater detail in the chapters on natural immunity. Apart from them, certain factors influence the susceptibility of an individual. They are the following:—

1. ***Age:***—Infants, owing to the non-development of their resisting powers, are susceptible to certain diseases, such as measles, diphtheria, whooping cough, etc. Old subjects, owing to enfeeblement of their resisting powers, are prone to attacks of pneumonia and other diseases.

2. ***Habits:***—Sedentary occupations, residence in ill-lit and ill-ventilated quarters, predispose to such an infection [illegible] tuberculosis. Uncleanliness, dirty habits and insanitary and unhygienic [illegible] make a person more liable to attacks of many infectious diseases.

3. ***Diminished vitality:***—This may be local or general.

(a) **Local:**—

Trauma:—Local injury to bones predisposes to such infections as osteomyelitis and tuberculous joint disease. Local injury to skin is one of the chief factors which render invasion possible by breaking the continuity of the epithelial layers. The degree of trauma, however, varies with different organisms. For example, it must necessarily be extensive for gas gangrene, but it may be very minute or even microscopical for syphilis.

(b) **General:**—

(i) *Poverty* leads to debility and debilitated and enfeebled subjects are particularly liable to acute infections, such as pneumonia, dysentery and tuberculosis.

(ii) *Constitutional diseases,* e.g., Diabetes and chronic Bright's disease. Such persons are very prone to infection by various kinds of pathogenic bacteria. A diabetic often suffers from pyogenic coccal infections of the skin, such as carbuncles and furuncles, while gangrene supervenes on slight injuries. Tuberculosis, pneumonia and other pulmonary diseases set in very easily and are frequently the terminal infections which prove rapidly fatal. A person suffering from chronic Bright's disease, is more prone to erysipelas and other Streptococcal infections than a healthy person.

(iii) *Certain infectious diseases:*—One infection may devitalise the system so much as to allow another infection to establish itself. It is for this reason that tuberculosis is commonly flared up after influenza, measles, whooping cough, etc.

(iv) *Vitamin deficiency:*—The lack of vitamins, particularly the anti-infective A and D varieties, in the dietary, is likely to increase the susceptibility to infections.

DEFENCES OF THE BODY AGAINST BACTERIAL INVASIONS

This can be discussed under two groups. *General or non-specific factors,* which are available for the resistance to any infection and such non-specific reactions may suffice when the number of organisms is small; and *specific factors,* which are created only in response to specific infections.

GENERAL OR NON-SPECIFIC FACTORS (Vide also the chapter on Natural immunity). These include the following:—

1. ***Intact, healthy skin and mucous surfaces:***—The unbroken surfaces of the body have a considerable protective action in preventing the entrance of micro-organisms. Any breach in the integrity will lower the resistance and favour the invasion by micro-organisms. Whereas a healthy condition of the mucous membranes prevents entrance of any germ, a catarrhal condition will upset the equilibrium and pave the way for the invading bacteria. It is an important fact that a tissue, in the stage of

catarrhal inflamation, is always in a state of lowered resistance and therefore cannot act as an efficient barrier to the invasion of other bacteria. Thus catarrh of the respiratory tract, produced during an attack of common cold, predisposes to the development of cerebrospinal fever. Similarly, catarrh of the respiratory tract in measles, whooping cough and influenza is often followed by a tuberculous infection.

2. ***Secretions and excretions of the body:***—They also play a part in eliminating the invading bacteria, by washing them out mechanically or by producing a destructive action on them, owing to the presence of certain enzymes.

3. ***Various reflex phenomena,*** such as coughing, sneezing, etc., play some part in warding off the bacteria which have already gained entrance into the body.

4. ***Rise of temperature:***—Febrile condition, generally induced by infection, is probably to some extent, a protective mechanism. The raised temperature is not only injurious and harmful to bacteria but it also produces a beneficial effect, owing to the associated increase in the pulse rate and consequent quicker circulation which leads to flushing out of tissues and elimination of toxins. Hence it is not desirable to reduce the febrile reaction too much in all cases by artificial means.

5. ***Phagocytosis:***—This is the process by which bacteria are destroyed by the leucocytes and tissue cells. The cells ingest the bacteria and destroy them by means of ferments. The most active phagocytic cells are the neutrophilic polymorphonuclear leucocytes which are concentrated in large numbers at the site of the lesion. Such a reaction is the result of a chemiotactic stimulus to the bone-marrow provided by the bacterial infection.

6. ***Bactericidal substances in blood serum:***—Blood serum contains certain bactericidal substances, such as alexin, β-lysin and others, which are normally present and tend to resist bacterial infection (Vide chapter on natural immunity).

SPECIFIC FACTORS:—Introduction of bacteria and their toxic products leads to the formation, in the inoculated animal (if the animal survives the effect of the inoculation), of certain immune substances which have the power to destroy or neutralise the inoculated material. The substances which on injection into the tissue of an animal give rise to the development of antibodies are known as *antigens* and the immune substances which are developed in the living body in response to the presence of antigens are called *antibodies*. The serum of the animal, which contains the antibodies is called an *immune serum* or *antiserum*. Similar results are obtained when an individual recovers from a disease. The antibodies so produced, circulate in the blood stream [illegible] are specific for that particular disease. Owing to this specificity, they [illegible] great value in the diagnosis of those diseases. The antisera may be [illegible] kinds (a) *antitoxic* and (b) *antibacterial*.

(a) **Antitoxic sera:**—These are produced in cases of infections by bacteria which produce exotoxin. The resulting antibody is called *antitoxin*.

(b) **Antibacterial sera:**—These contain antibacterial antibodies which are produced as a result of inoculation with the bacteria which produce endotoxins. Two classes of such antibodies are recognised.

(i) A class in which the interaction with antigen in saline is demonstrable by the use of the antibody alone, e.g., *agglutinins* and *precipitins*. The difference between agglutination and precipitation reactions however depends on the use of antigens in two different states, particulate and soluble.

(ii) A class in which the presence of a labile substance in the serum, called *complement*, is necessary to complete the reaction, e.g., bacteriolysins. Antibodies which require complement for their demonstration are designated by the generic name *immune bodies or amboceptors*. The nature of these immune bodies may be of various kinds and they are named according to the nature of their action, e.g.:—

1. *Bacteriolytic immune body*—when it leads to lysis of bacteria.
2. *Hæmolytic immune body*—when it leads to lysis of erythrocytes.
3. *Opsonic immune body*—when it leads to phagocytosis.
4. *Complement-fixing body*—when the combination takes place with or without lysis of the antigen.

PATHOLOGICAL EFFECTS OF BACTERIA

After pathogenic bacteria have gained entrance into the body, they begin to multiply rapidly and produce poisons which may act both upon the tissues around and also upon the body generally. The bacteria inflict injury chiefly by originating poisonous substances, the nature of which has already been discussed on p. 38-39.

The bacteria may remain localised, producing either a marked local reaction, or little local reaction with marked general effects. The bacteria may also find their way into the blood stream, via the lymphatics and veins, and then may settle in other parts of the body, where they multiply and produce lesions.

Certain terms in connection with the generalisation of bacteria or their toxic products are discussed below:—

1. **Bacteriæmia:**—This is a condition in which there is a mere presence of the organisms in the blood. The organisms are few in number, they fail to multiply in the blood and are soon destroyed. The best example of bacteriæmia is the early period of *typhoid fever*. The term, therefore, signifies the temporary existence of the specific micro-organisms in the blood stream and the condition being regarded as incidental to, but not co-extensive with, the infection.

2. ***Septicæmia:***—This is a condition where the organisms actively multiply in the blood stream and tissues and produce toxins. This is a much more serious condition. The bacteria which are notable for the production of a septicæmic condition are *Streptococcus hæmolyticus, Staphylococcus pyogenes (in puerperal and post-mortem or post-operative wounds), D. pneumoniæ (Pneumococcus), N. meningitidis (Meningococcus), B. anthracis, P. pestis (Plague bacillus), etc.* These organisms break the tissue barrier at the site of local infection and invade the blood stream.

3. ***Pyæmia:***—This is a condition in which septicæmia is complicated by the formation of multiple abscesses in different parts of the body. These metastatic abscesses originate as a result of detachment of small portions of infected thrombi, containing bacteria, and their subsequent lodgment in remote capillaries. According to the origin of these infected thrombi, pyæmia may be divided under three groups:—

(a) *Arterial pyæmia*, e.g., infective or ulcerative endocarditis.

(b) *Portal pyæmia* (suppurative pylephlebitis), e.g., in suppurative appendicitis and suppurative piles.

(c) *Systemic venous pyæmia*, e.g., in osteomyelitis, cellulitis and septic uterus (puerperal).

4. ***Toxæmia:***—It is a condition where the products of bacterial growth (toxins), without the bacteria themselves, circulate in the blood stream from a localised infection. Hence these diseases are characterised by the absence of bacteriæmia. The most important examples are *tetanus, diphtheria, cholera and bacillary dysentery.*

5. ***Sapræmia:***—This is a variety of toxæmia. It is called septic intoxication and is due to the absorption of poisonous products from those organisms which are capable of attacking dead tissues only (saprophytes), e.g., *gas gangrene* and *tetanus.*

In this connection, it should be remembered that after generalisation, the organisms or their toxins show special predilection for particular tissues. This ***selective localisation*** however varies with different organisms, e.g.:—

E. typhi for small intestine.

N. meningitidis for subarachonoid space.

D. pneumoniæ for lung parenchyma.

Diphtheritic toxins for peripheral nerves, heart and kidneys.

Tetanus toxin for motor nerve cells (tetanospasmin) and for red cells (tetanolysin).

Botulinus toxin for cranial motor nerve cells (Oculomotor and Vagus).
Again, micro-organisms of the same species may show a considerable variation in their tissue affinities. For example, while some Streptococci affect the skin producing erysipelas, others affect the throat causing scarlet fever and tonsillitis, whilst still others involve the heart, the gall bladder and the stomach, giving rise to endocarditis, cholecystitis and gastric ulcer respectively.

PATHOLOGICAL CHANGES PRODUCED BY BACTERIA

These may for convenience be arranged in a tabular form as follows:—

1. **LOCAL:**—When the poisons remain mainly confined to a particular spot. The character of such local lesions may be (a) inflammatory tissue reaction (cellular reaction) and (b) degeneration and necrosis. Such local reaction in tissues, in certain circumstances, is a specific response which is so characteristic as to be diagnostic of the condition. This is particularly seen in infective granulomas, such as tuberculosis, syphilis, leprosy, actinomycosis and various other conditions.

2. **GENERAL:**—When the bacteria or their poisons are diffused through the circulation, they produce changes which may be specific, reactionary and general systemic.

(a) ***Specific changes:***—These depend on the selective localisation.

(b) ***Reactionary changes:***—These are manifested in certain organs, such as the *bone-marrow* and *the reticulo-endothelial system*. These effects are particularly seen in the alterations in the circulating blood, such as leucocytosis with neutrophilia, anæmia, monocytosis and formation of various immune bodies.

(c) ***General systemic changes:***—These are the following:—

(i) **Injury to secreting glands,** such as kidneys and liver. It is for this effect, that the urine becomes high coloured, scanty and concentrated. In cases of severe damage to the kidneys, evidences are found in the presence of albumen and tube casts in the urine. The secretions of the liver, stomach and the intestines are also greatly hampered, producing anorexia, constipation and various other digestive disturbances.

(ii) **Involvement of vital organs:**—The most important vital organs which are affected are the heart, suprarenals and the nervous system. The weakness of the heart and the fall of blood pressure after an infection are all due to such involvements.

(iii) **Fever:**—A rise in temperature is a very constant accompaniment, the degree of fever always depending on the character and intensity of the poison and the susceptibility of the heat-regulating mechanism of the affected individual.

CHAPTER X

IMMUNITY

DEFINITION:—Immunity is a term which broadly denotes non-susceptibility to disease. In bacteriology, it is applied to mean a resistance which an organism develops, either as a result of inheritance or of having acquired it some time during life, either by passing through an attack of the disease or through an artificial inoculation. It is known from very ancient times that a person who gets an attack of smallpox, does not suffer from the disease a second time in his life. Once a man survives an attack of cholera, typhoid fever or diphtheria, he may remain free from the risk of a second attack of the same disease. The resistance which a person acquires after recovery from an infective disease is due to the development of immunity.

VARIOUS METHODS OF PRODUCTION OF IMMUNITY:— For purposes of convenience, the various aspects of immunity have been summarised in a tabular form.

Immunity

Natural (Inherited or Genetic) | Acquired

Species | Racial | Personal or individual | Active | Passive

Specific | Non-specific | By inheritance | By artificial means

General | Local | By foreign proteins and heavy metals | (i) Serum therapy | (ii) Bacteriophage

By an attack of the disease | By repeated and frequent exposures to small minimal doses of in- | By some artificial means | Cutaneous | Intestinal

Anti-virus | Bili-vaccine

Injection of bacteria

(i) Living and highly virulent but in sm
(ii) Living but with reduced virulence
(iii) Dead (vaccines)

Injection of toxins

lethal doses

NATURAL IMMUNITY

This means spontaneous resistance which is possessed by every animal to fight against the harmful effects of micro-organisms and their poisons. Every animal naturally possesses a large amount of inherent forces by which its body can oppose noxious influences of whatever kind that may disturb its ordinary smooth working. The sum total of such defensive forces may be defined as natural immunity. This may depend on the following:—**1.** Species, 2. Race and 3. Personal or individual factors.

1. ***Species immunity:***—It is observed that certain diseases which are common in human beings are practically unknown amongst animals. For example, animals do not suffer from the following diseases, such as typhoid fever, cholera, scarlet fever, gonorrhœa and syphilis, which are exclusively human diseases. Conversely, there are diseases amongst animals, which do not spontaneously attack man. Thus, human beings enjoy immunity against rinderpest and to a less extent, against chicken cholera. Again, it is seen that amongst the same species, some animals are very susceptible to certain diseases, whereas others are resistant to them, e.g.:—

i. Rats are immune to diphtheria, whereas guinea-pigs are very susceptible.

ii. Dogs, sheep, goats and horses are immune to tuberculosis but guinea-pigs are very susceptible to it (particularly to the human type.).

iii. Pigeons are immune to pneumococcus infection, but white mice or rabbits are very susceptible to it.

iv. Alligators, lizards and turtles are immune to tetanus.

v. Rats, dogs, fowls, frogs and crocodiles are immune to B. anthracis and Cl. tetani.

vi. Hedgehogs and mongooses are resistant to snake venoms.

2. ***Racial immunity:***—Apart from the species immunity, separate races within the same species, display differences in their susceptibility to certain diseases. Amongst human beings, the Jews have more resistance against tuberculosis than the Negroes. In our country, it is often seen that men residing in the Himalayas, possess little or no resistance against tuberculous infection. When they are once infected, they die very quickly, with rapidly developing lesions. Amongst animals, while ordinary sheep are very susceptible to anthrax, the Algerian variety is very resistant to it.

3. ***Personal or individual natural immunity:***—Besides the species and racial immunity, there are certain defensive mechanisms which are common to all animals. These can be discussed under the following heads: —

A. Physical.

(i) *Integrity of the investing layers of the body*—The investing layers of our body are the skin and epithelium, and so long as these are intact, they offer an almost complete barrier to the entrance of bacteria into the deeper tissues. The acid reaction of the sweat constantly serves as an inhibitory agent against the pathogenic bacteria which may be lodged on the skin surface, thus serving the purpose of auto-sterilisation; while the hard squamous cells in the superficial layers of the epidermis, constantly undergoing keratinisation, act as an impenetrable barrier against the entrance of the organisms.

(ii) *Secretions and excretions of the body*—These form the second line of defence to the entrance of the organisms. They constantly bathe the epithelial surfaces and thus maintain the healthy condition of the skin and mucous membranes. The secretions from the mucous glands (mucin or mucus) are also very useful. They are present in various mucous membranes and act as a protective coat by offering a barrier against the invading bacteria. They act mechanically by entangling the invading organisms and subsequently getting rid of the intruders. It will be seen later that the presence of lysozyme, a ferment-like substance, may help, to a certain extent, to prevent the settlement of any bacteria, particularly saprophytic ones, on conjunctiva and other mucous membranes.

(iii) *Ciliated epithelial cells*—These produce a current and such physical movement can ward off the invading bacteria or offending materials. The best example of this is the ciliated epithelium of the respiratory tracts.

B. Chemical.

(i) *Gastric Juice*—The free hydrochloric acid (the normal figure varies from 0·02 to 0·15 per cent.), secreted by the gastric mucous membrane, is highly bactericidal and thus acts as a strong protective barrier. Acidity of the gastric juice has, therefore, a great value in the prevention of many diseases like cholera, enteric fever, tuberculosis, etc.

(ii) *Lysozyme*—This name was given by Fleming (1922) to a ferment-like bactericidal substance, which is found in all tissues and in all secretions, except in urine, sweat and cerebrospinal fluid. It is specially found in greatest concentration in tears and cartilage. This substance does not dissolve all species of organisms but produces its bactericidal effect on certain types of saprophytic bacteria, more particularly, a type of saprophytic micrococcus, called by Flemi[illegible] *Micrococcus lysodeikticus.* This organism is particularly used to de[illegible]te the action of lysozyme. In addition to the above organisms, en[illegible]i are specially susceptible to lysozyme. Owing to the presence of [illegible]erful ferment in the secretions which bathe the mucous surfaces [illegible]yes, nose and mouth, the saprophytic invaders are unable to get a suitable lodgment there. But it is doubtful how far lysozyme-action can extend to organisms of the more parasitic type.

C. Cellulo-humoral:—This is a variety of defensive mechanism which is present as an inherent and primitive function in the cells and fluids of the body.

1. *CELLULAR:*—In every scale of creation, there is always present a sufficient provision for defence and regeneration, otherwise no species would live and survive. In an unicellular organism, this is an inherent duty of the protoplasmic mass which constitutes the body of the organism. For instance, when a small portion of the cytoplasm of such an organism is destroyed, leaving the nucleus intact, the injury is quickly repaired by a closing together of the uninjured portion of the protoplasm. When any bacteria or foreign particles happen to come in contact with its body, producing an injurious influence, the same is at once engulfed in the mass of the cytoplasm with the help of pseudopodia. Thus, the various properties such as chemiotaxis, phagocytosis, adaptation and healing, which are necessary for the existence of life and its defence, are all present in one and the same structure, viz., the protoplasm of the organism. With the evolution of the creation, when we examine the lowest forms of multicellular organisms, we observe some differentiation and division of labour among the various cells constituting their bodies (Fig. 66). For instance, the outer layer of cells remains protective, as an outer line of defence against external invasions, whereas the inner layer, which becomes invaginated, is entrusted with the digestive functions. Between these two layers of cells, there is found a special group of cells, the mesodermal cells, lying irregularly scattered, and with very marked powers of motility and phagocytosis. They represent the lowliest forms of leucocytes, the wandering phagocytic mononuclear cells and the connective tissue cells and are the principal agents for internal defence and repair. If any living cell be destroyed or any injurious agent gets an access into the body, those cells in the immediate neighbourhood spread out their pseudopodia with a view to engulf the injurious agent, remove the debris and fill up the gap by rapid multiplication and growth. In the higher scale of creation, with the development of the vascular system, these various defensive cells are distributed in a regular order in different groups and in different localities, so as to be able to serve the best interests of the individual. Compare the conditions of aboriginal tribes-men in Central Africa with the highly organised system of any modern power of Europe or America. While in the former the defensive

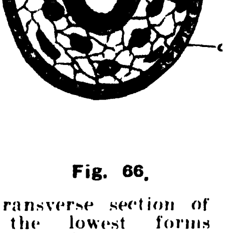


Fig. 66.

A transverse section of one of the lowest forms of metazoa showing the functional differentiation of cells (Diagrammatic).

ec, ectoderm; en, entoderm; m, mesodermal cells; pl, a plasmodium mass formed by the fusion of wandering mesodermal cells to engulf the foreign body f.b.

mechanisms are less effectual and disorderly, in the latter, the same are highly organised and very effective and strong, not only with the modern scientific weapons of defence, but also with the rapid transport facilities and means of communication.

THE CELLS FOR DEFENCE:

(i) *In the circulating blood :*

(a) *Granular*—Neutrophil and eosinophil cells.

(b) *Non-granular*—Lymphocytes and hyaline leucocytes.

(ii) *In the tissues:*

(a) *Wandering cells*—These are actively motile with amœboid movement and possess the power of phagocytosis. They are mononuclear cells.

(b) *Connective tissue corpuscles*—These are somewhat fixed and act as a sort of a ready reserve for repairing an injury.

(c) *Sessile histiocytes*, lining the capillaries of the liver, spleen, bone-marrow, lymph glands, etc. They are possessed with great power of phagocytosis and production of powerful antibodies.

A few words about their functions and modes of action may be mentioned here. In the defensive mechanism, their work may be as follows:—

(1) *Mechanical:*—This is carried on mainly by a process of phagocytosis.

(2) *Biochemical:*—

(i) *Ferments and enzymes:*—These are liberated in the serum when the leucocytes are disintegrated, as for example, in an area of inflammation. They are proteolytic in nature and liquefy the exudate and the damaged and injured tissues before repair is possible.

(ii) *Production of opsonins and other immunity factors:*—These diffuse into the circulating blood and are detectable in the serum.

(3) *Reparative:*—This comes very late.

II. *HUMORAL:*—Apart from the various physical, physico-chemical and cellular agencies, there are various basic defensive mechanisms inherently residing in our tissues and fluids. These are:—

(i) BACTERICIDAL ELEMENTS IN THE BLOOD:—Lord Lister (1880-81) found that ox-blood, when shed aseptically from the animal, keeps for long and that even milder grades of contamination will have no effect in producing a detectable growth. On the contrary, if a single bacterium gets into milk, an abundant growth will take place. This experiment demonstrates that the blood has an inherent property of destroying organisms, due to the presence of bactericidal or growth-[illegible]ting elements in the serum. The following substances have been found in the serum to produce the bactericidal action.

(a) *Leukins and Plakins:—Leukins* are bactericidal substances derived from the extracts of leucocytes. They are heat-stable bodies. They have a marked bactericidal action, specially on the saprophytic spore-bearing group. *Plakins* are also similar substances obtained from platelet extracts. These substances cannot be increased by active immunisation.

(b) *β-Lysins (Petterson):*—This term is given to a heat-stable (less than leukin) bactericidal substance present in normal serum to differentiate it from *leukins* and *alexin (a-lysin)*. The substance has a marked bactericidal action on the organisms of the anthrax-subtilis, and also the proteus groups. There is no evidence that *β*-lysin content of the serum can be increased in amount by specific immunisation.

(c) *Alexin* or *a-lysin:*—Buchner gave the name *alexin* to another bactericidal substance present in normal serum which is heat-labile, *i.e.*, destroyed by heating to 55°C. for 30 minutes. The organisms which are readily susceptible to the action of alexin are the typhoid-colon-dysenteriæ group. Other organisms that are susceptible to its action are Vibrio comma (V. choleræ), Pseudomonas æruginosa (B. pyocyaneus), Diplococcus pneumoniæ and Streptococcus. This substance however can be increased by specific immunisation.

The following is a table showing the differences between these various bactericidal substances:—

	Alexin or *a*-lysin.	*β*-lysin.	Leukins.
Source.	Normal serum.	Normal serum.	Extracts of leucocytes.
Heat-resistance.	Heat-labile.	Less heat-stable than leukins.	Heat-stable.
Readily susceptible organisms.	Typhoid-colon-dysenteriæ group.	Anthrax-subtilis group.	Saprophytic spore-bearing aerobic group.
Effect of immunisation.	Increased by specific immunisation.	No effect.	No effect.

(ii) OPSONINS:—In normal serum, there is a substance which is able to sensitise micro-organisms and make them more suitable for phagocytosis by the poly-nuclear leucocytes. To this substance, which is thermo-labile, Wright and Douglas (1903) gave the name *opsonin*.

(iii) AGGLUTININS:—This is a substance present in the normal serum to a certain extent and shows a considerable degree of specificity. Their main action is to produce clumping of bacteria and thus help bacteriolysis.

ACQUIRED IMMUNITY

This type of immunity is developed after an attack of a disease or by artificial means. It may be, either (i) *active*, when the cells of the body take an active part in the production of immune bodies; or (ii) *passive*, when the cells of the body do not take any active part in the production of immune bodies which are only transferred passively from one individual to another.

ACTIVE IMMUNITY

ACTIVE IMMUNITY is a term which denotes a resistance imparted to the individual by the activity of the various defensive forces existing in his body. This may develop either from an actual attack of the disease or by artificial means. When once developed, it usually tends to persist for a long period. The elements of the body which are responsible for the production of these protective substances remain in a very highly sensitive and excitable state, so that, as soon as there is a chance for any second attack of the same disease, they begin to throw out into the blood and tissue fluids, antibodies, in an explosive way, resulting in the complete arrest of the progress of the infecting agent. Active immunity may be specific and non-specific.

Specific active immunity may again be divided into (a) *general* or (b) *local* (Besredka). The latter is only applied to skin (anti-virus) and intestinal mucous membrane (bili-vaccine) at present. This type of immunity is acquired against a certain specific organism or infection.

Non-specific active immunity is a resistance which is developed from a greater mobilisation of the normally existing antibodies, as a result of the stimulation of the antibody-forming elements in the system. This may be induced by the introduction into the body, of substances, such as foreign proteins or heavy metals. In this general mobilisation of defences, any infection which may be present at the time, may be more effectively dealt with.

SPECIFIC ACTIVE IMMUNITY, GENERAL

METHODS OF PRODUCTION:—

1. *By an attack of the disease.*
2. *By repeated and frequent subminimal infections.*
3. *By artificial process.*

1. ***By an attack of the disease:***—Examples of such active acquired immunity are afforded by all those cases in which recovery from a single attack of an infective disease confers absolute or relative immunity against a recurrence of the same infection. The following are some of the diseases in which active resistance is produced as a result of recovery from a typical attack of the disease; smallpox, enteric [illegible] diphtheria, scarlet fever and

cholera. Pneumonia and influenza, on the contrary, are the diseases in which an attack confers little or no immunity. When a person is attacked with any disease, all the cells and fluids of his body acquire newer and greater powers of attacking the invading organisms. These are developed from the various natural defensive forces which already exist in his body. When the person recovers, all the protective powers persist in a very much augmented state, to a greater or less extent, throughout the rest of his life.

2. ***By repeated and frequent subminimal infections:*** With this mode of infection, the person does not suffer from the symptoms of an actual disease. These infections are so light in severity and transient in duration as to escape detection, but they may have a considerable immunising value. Commonest examples of such immunity are seen in the case of laboratory workers, hospital staffs, nurses and so on. This is very important from the point of view of public health, as mass immunity is developed in this way. If one takes a large number of unselected young children from slums, as well as, from well-to-do persons, he will find that the percentage of children who are non-susceptible to diphtheria, *i.e.*, giving a negative Schick reaction, will be greater in the poorer class than in the well-to-do class. The production of such Schick-negative cases can be explained by supposing that the children of the poorer class of people have greater chance of coming in intimate contact with either carriers or sufferers and thereby making them liable to frequent risks of small doses of infection which built up and gradually increased the amount of immunity. It is in this way that a large number of children amongst poorer classes is naturally protected from many infectious diseases and, even if they suffer, the disease is milder in form. Similarly, it may be explained why some men, although in poor circumstances, are better able to resist many infections than gentlemen in society. A *dome* (menial) in the post mortem room can resist wound infections which would prove certainly fatal to any one of us.

3. ***By artificial process:***—First classical example of inducing this sort of immunity was the smallpox inoculation which was first scientifically introduced by Edward Jenner.

Story of the evolution of smallpox inoculation:—In India and China, it was known from very ancient times that the inoculation into children, of matter taken from the smallpox pustule, protected them against the severe disease. This knowledge gradually spread westwards, till it reached Arabia and Constantinople. It was from this place that Lady Mary Wortley Montague learnt the method of direct inoculation of smallpox virus and introduced the same to England on her return, in the middle of the eighteenth century. This, however, led to an increase in the incidence, because, although the disease was very mild in the inoculated persons and although such men were immunised, it led to much carelessness on the part of the patients and hence the disease was indiscriminately scattered to those who came in contact with the inoculated men. Thus the method fell into disrepute and was ultimately given up for the time being.

This matter was however revived by an English physician, Edward Jenner (Fig. 67), and it was he who first noticed that cow-pox conveyed to man protected him against smallpox. This idea, derived from the chance remark of a dairy maid "I cannot take smallpox for I have had cow-pox," impelled him to make a direct test of its correctness. He published his methods and results before the Royal Society of London in the year 1796 and proved beyond doubt the efficacy of smallpox vaccination in the control of a dreadful disease which was often fatal and very disfiguring. Now we know that he employed a method by means of which the virus of smallpox, whose real nature is not yet known, was attenuated by passage through another species of animal, viz., the cattle. Thus the vaccination conveyed to the inoculated person a modified form of smallpox, rendered mild and harmless by its passage through the cow.

The vast development of the modern ideas about vaccine therapy and protective inoculation originated in 1880 from Louis Pasteur (Fig. 68), a professor of Chemistry, in trying to find out the cause of poultry diarrhœa in the neighbourhood of Paris. He found out the etiological agent of this fowl diarrhœa to be a minute bacillus and noted that when cultures grew very old, inoculation from such cultures did not kill the fowl. When these fowls were, however, subsequently used for inoculation purposes with a powerful fresh virus, he found, to his surprise, that they did not succumb like the other uninoculated ones. With the characteristic genius he possessed, he conceived the full significance of this observation, instead of finding fault with his experiments or assistants. He [illegible] that, on keeping, the cultures

Fig. 67.

Edward Jenner (1749-1823).

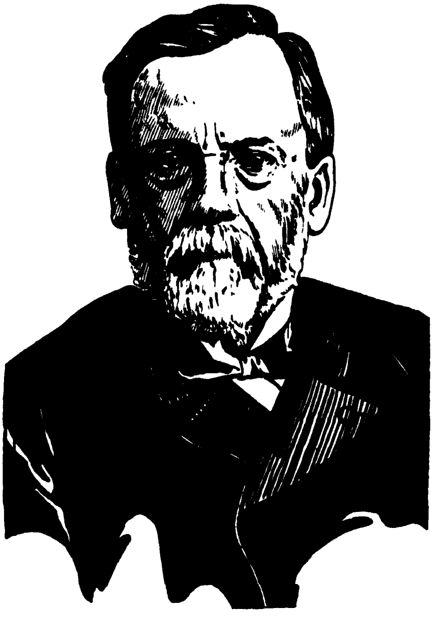


Fig. 68.

Louis Pasteur (1822-1895).

had become attenuated and the inoculation with this attenuated virus had conferred immunity and protection to the chicken against subsequent infection. Pasteur also found out a protective inoculation against anthrax and rabies, similarly, by the use of a living attenuated virus.

Methods of producing artificial immunity:—

1. *INJECTION OF BACTERIA OR ANY OTHER INFECTIVE AGENT:—*

(a) *Living but with reduced virulence* (Use of attenuated organisms):—Pasteur's methods of prophylaxis against rabies and anthrax are the examples of the use of living attenuated organisms in the production of active immunity. The various methods of attenuation are discussed on p. 95.

(b) *Killed organisms:*—The common *vaccines* are the examples. This will be described in detail in chapter XII. Anti-typhoid inoculation is the best example of the use of killed cultures for active immunity (prophylactic).

2. *INJECTION OF TOXINS:*—The use of bacterial toxins in the method of producing active immunity is afforded by the modern methods of immunisation against scarlet fever and diphtheria.

To sum up:—The most important examples of specific general active immunity, as applied practically, are the following:—

1. *Prophylactic inoculation of sheep and cattle against anthrax.*
2. *Jennerian vaccination against smallpox.*
3. *Inoculation against hydrophobia.*
4. *Toxin-antitoxin or anatoxin (toxoid) inoculation against diphtheria and scarlet fever.*
5. *Prophylactic vaccination against cholera, plague and enteric fever.*

LOCAL IMMUNITY

(SPECIFIC ACTIVE IMMUNITY, LOCAL)

The idea of local immunity originated from Besredka of Paris (1919-24). Two types of local immunity are commonly known at present.

1. *For skin—Anti-virus.*
2. *For intestinal mucous membrane—Bili-vaccine.*

ANTI-VIRUS:—This name was given by Besredka to an inhibiting thermostable substance, contained in the filtrate of a broth culture of certain bacteria which, when applied in the form of a poultice or compress over the shaved skin, prevents the infection of that area by a virulent strain of the particular organism. According to him, an essential and cardinal feature of this form of immunity is the absence of antibody formation in the blood and therefore, the immunity developed is strictly a local one.

Besredka's experiment:—He took 1 c.c. of a 24 hours' broth culture of Staphylococci and injected it into the skin of the abdomen of a guinea-pig. This produced a rapidly spreading œdema and gangrene, and the sloughing ulcer took a long time to heal. But when he applied a compress of heated broth culture of Staphylococcus to the shaved skin of the animal on the previous day and injected a virulent strain of the organism subsequently into that area, he noticed only a small and superficial lesion, perhaps an abscess, and the healing was very quick. He then began working with older cultures and found that by filtering an 8 to 10 days' old broth culture of Staphylococci, he was able to obtain a substance which produced similar effects. It was to this substance that he applied the name anti-virus.

Mode of action of anti-virus:—In actual infection, the organisms first exert their action on the local reticulo-endothelial system of cells which form the first line of defence. When these are paralysed or their defensive capacity is exhausted, the organisms penetrate further and cause more damage, either locally or generally. The anti-virus appears to immunise these receptive reticulo-endothelial cells against the action of the bacteria. They become desensitised, as it were, and the injurious element, when subsequently introduced, finds it difficult to effect a foothold into the tissues which are thus capable of dealing fully with it. According to Besredka, the local immunity so developed is mainly confined to the area so treated, though the neighbouring portions of the cutaneous surfaces may be immunised to a less degree. This is not due to the formation of antibodies circulating through the blood, but to a superficial lymphatic spread.

Preparation:—An eight to ten days' bouillon culture is filtered and heated to reduce its toxicity. The filtrate is applied as a wet dressing. The anti-viruses that have been effectively used as therapeutic agents, have been obtained from (i) Staphylococci and (ii) Streptococci.

Uses:—Anti-virus is chiefly used (i) in the inflammation of the skin caused by Streptococci and Staphylococci and (ii) in erysipelas. Besredka has also produced a solid immunity in guinea-pigs against anthrax infection, by applying Pasteur's attenuated vaccine over the shaved abdominal skin of the animal.

BILI-VACCINE:—These are substances prepared from the growth of certain bacteria on solid media and are taken *per os*, for protection against infection by similar organisms through the intestinal mucous membrane. The underlying principle is similar to that of the use of anti-virus for skin immunity. In cases of typhoid, cholera and dysentery infections, the organisms first attack the local defensive cells in the mucosa of the intestines, which form the first and the most important line of defence. It is only when they are overcome, that the invading bacteria or their toxin can penetrate deeper and attack the general system. According to Besredka's idea, the real protection should consist in increasing the resistance of the cells of the local mucous membrane, and this is possible only by the oral administration of vaccines made from these organisms. The preliminary administration of bile is supposed to facilitate the absorption of the organisms or their toxins, due probably to the formation of [illegible] microscopic erosions in the mucous membrane. Thus, a good deal of protection is said to be conferred on the organism as a whole, by an increase in the local resistance of the tissue elements which constitute their natural avenue of infection. It is for this reason that bili-vaccines are employed for prophylaxis against those infections which usually enter the body through the gastro-intestinal tracts, e.g., Eberthella typhi, Vibrio comma, Eberthella dysenteriæ and others. One of the essential features of such oral vaccination is, according to Besredka, a total lack of formation of the antibodies in the body of the immunised person.

There has been a good deal of controversy as to the real nature of this sort of local immunity. Although Besredka is of opinion that the immunity obtained is strictly local and is of a specific nature, it has not been uniformly upheld by other workers. The latter believe that this form of local immunity is in no way specific but only a manifestation of the local mobilisation of the defensive forces of the body. According to them, it is similar in action to the immunity conferred by the use of non-specific substances. A large number of tissue histiocytes is attracted to the area in question—a reaction which is excited by substances other than anti-virus or bili-vaccine, such as meat extracts, peptones and mild irritants. The large number of histiocytes which wander into the irritated area constitutes a sufficient barrier to prevent an infection. Such a protection, of necessity, should last only for a period during which this histiocytic response persists. It will be seen that, if the reaction induced in this way is strong and repeated, it may lead to a general mobilisation of the defensive forces of the body in addition to the local immunity. Under such conditions, antibodies have been demonstrated in the blood.

ACQUIRED IMMUNITY BY NON-SPECIFIC SUBSTANCES

We have seen that for a particular microbic infection, it is possible to obtain a specific vaccine which helps the patient to counteract the effects of that particular organism which attacks him. All the beneficial results of such a remedy were supposed to be due to a specific effect on the course of infection by the specific bacteria, until lately, when some doubt has been cast on this specificity. It is now maintained with very good reasons, that non-specific substances may also produce an increase of immunity reaction of the body to ward off different kinds of infections. That the introduction of non-specific foreign substances like broth, peptone and even urine, into the peritoneal cavity of an animal can alter the local defences of the peritoneum, so that the animal is subsequently able to withstand a considerable amount of infection, has been known from a long time. For instance, Pfeiffer and Issaeff, in 1894, found that a lethal dose of cholera vibrio injected intraperitoneally, is unable to kill a rabbit into whose peritoneal cavity some sterile nutrient broth had previously been injected. Although the exact mechanism of this increased defence was not exactly known at the time, it was suggested that such intraperitoneal inoculations gave rise to a local leucocytosis, as a result of which, the cholera vibrios injected subsequently, were more rapidly dealt with, either by direct phagocytosis or by the effect of the bactericidal substances liberated by the broken down leucocytes. This phenomenon of Pfeiffer and Issaeff laid the foundation of the modern idea about the production of immunity by non-specific means.

Mechanism of action of non-specific protein therapy:—The local cell elements which are concerned with the defensive mechanism, respond to the injection of these non-specific substances by an increased proliferation and consequently, by a greater mobilisation to deal with the inoculated foreign substance which cannot remain inside the body unattended to for any length of time. This acts as a sort of stimulus which in fact renders the local tissues more responsive and more alert. With suitable amounts of injection, a general stimulation of these antibody-forming elements may be induced, resulting in a considerable enhancement of the general defensive powers of the body. During such a period, when there is an exacerbation of the normal defensive powers of the body, any infective element which may be present in any part of the system, is also dealt with and disposed of. It should however be remembered that such a bout of increased defensive mechanism is only a temporary phase, lasting for about a week or 10 days, and that a further stimulation is necessary, either to maintain or prolong the period. It is for this reason that injections of non-specific substances are required to be repeated to obtain full therapeutic effects.

Examples of non-specific therapy:—

(a) Foreign proteins:—These include the following—(i) Bacterial vaccines; usually T.A.B. (typhoid and paratyphoid group of orgnaisms) and Coli (Escherichia coli) are employed; (ii) nucleins; (iii) peptones and proteins, including serum protein (normal and specific sera) and milk proteins (aolan, lactolan, yatren-casein, etc.).

(b) Heavy metals in colloidal state, e.g., silver (electrargol), copper (electrocuprol) and gold (sanocrysin and solganol).

Cases where non-specific protein therapy is done:—

1. In all cases of prolonged subacute pyrexia of unknown etiology, not responding to routine treatment.

2. Chronic infections of known or unknown causation.

3. Chronic arthritic and other kinds of inflammations, notably pelvic inflammations. It is thus extensively used in gynæcological practice for the treatment of tubal or parametric inflammations.

This non-specific protein therapy is particularly indicated where the foci of infection cannot be reached for culture and preparation of an autogenous vaccine or where the infection has lasted so long that the specific reaction is no longer sufficient for a cure and where changes in structure prevent sufficient circulation of antibodies. It appears that the lighting up of infection during the non-specific protein reaction, reactivates the healing mechanism.

Methods of administration:—If a sharp reaction is required, it is preferable to choose a bacterial protein and generally vaccines of Escherichia coli or enteric group (T. A. B.) of organisms are employed. As a rule, an initial dose of about 20 millions of each organism is given intravenously in 5 c.c. of sterile saline. Two to four hours before the injection, the patient should have a liberal carbohydrate food (glucose feed) and he should be in bed during the reaction. He should be encouraged to drink plenty of fluids and a small dose of codeine or adrenalin is given to shorten the reaction and lessen the discomfort. The injections are repeated at short intervals (2 to 4 days), as too long an interval may produce anaphylactic shock. Subsequent doses, however, depend to some extent on the reaction obtained.

If a more prolonged and less severe reaction is desired or if intravenous injection is impossible, 5 to 10 c.c. of sterile milk can be given intramuscularly. The chief disadvantage of this method is that it causes much discomfort and pain at the injected area. Usually 5 or 6 injections, given every third day, will suffice for a particular case.

Effects of "Protein shock" therapy (Fig. 69):—The immediate effect of the injection is the production of a sharp rise of temperature, attended with a severe chill and rigor, aches and pains in the body. The total duration of this reaction is usually 24 to 48 hours. This is followed by a brisk leucocytosis and a general stimulation of the reticulo-endothelial system, resulting in the production of antibodies in the serum, particularly the opsonins (tropins).

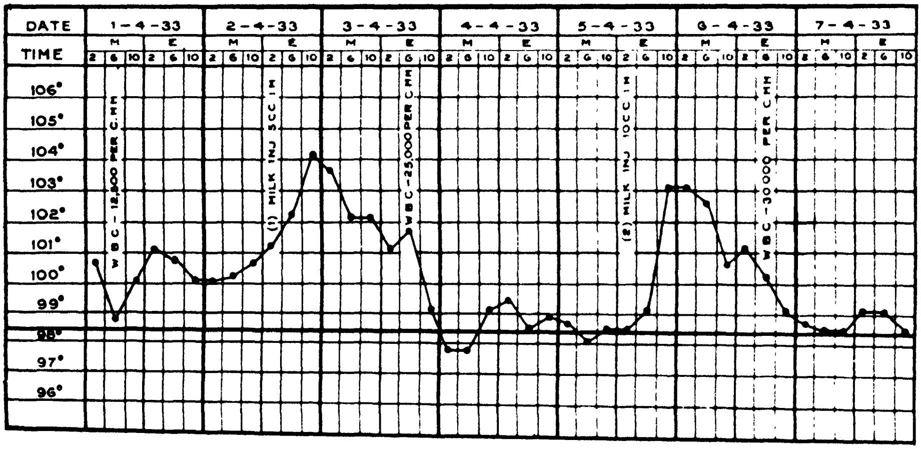


Fig. 69.

Temperature chart showing the effect of protein shock therapy in a case of chronic pelvic inflammation. Note the febrile reactions and leucocytosis after the intramuscular injection of milk (skimmed).

Contra-indications:—

(1) All forms of allergic and anaphylactic conditions.

(2) A low blood pressure with decompensated heart lesions.

(3) Acute organic disease of the kidneys.

(4) All forms of tuberculosis, as unsuspected, latent or quiescent tuberculous lesions may be flared up with disastrous results.

(5) Diabetes and (6) Pregnancy.

Remarks about injection of serum:—Many cases of prolonged subacute pyrexia of unknown origin are sometimes cured by the empirical use of anti-streptococcal serum, in most cases on an erroneous and imaginary diagnosis of the condition. The beneficial results in such cases are due entirely to marked non-specific effects of the serum proteins.

PASSIVE IMMUNITY

This type of immunity is produced in an animal by the injection of a serum from another animal which has been highly immunised by any of the methods of active immunisation. Such a serum supplies ready-made immune substances which are present in it in a preformed condition. The immunity is thus passively transferred from one individual to another. The recipient animal plays no active part in the production of these immune substances and for this reason, the process is spoken of as ***passive immunisation.***

The immunity conferred by this method lasts a comparatively short time, as the active constituents of the injected serum disappear gradually in about two to four weeks' time and the body cells are not educated to produce more. Active immunity, on the other hand, takes a fairly long time to develop and once developed, it persists longer, even to some extent for life, as the cells of the body have taken an active part in the manufacture of the antibodies and have retained their capacity to produce more, in response to fresh stimuli of the same kind. Owing to these reasons, active immunity is mainly used for prophylactic purposes and for the treatment of subacute or chronic diseases, whereas passive immunity is most useful as a curative agent in the treatment of acute diseases. In the latter conditions, where the necessity of immune substances is immediate and the patient is incapable of manufacturing such substances himself, owing to the paralysing action of the invading organism, the employment of passive immunity produces a remarkable benefit by tiding over the crisis.

Methods of production:—The various methods by which passive immunity may be conveyed are summed up in the following table:—

Passive immunity

By inheritance
By artificial means

Serum therapy
Bacteriophage therapy

Use of serum of an immunised animal
Use of convalescent serum

By inheritance:—Immunity of the [illegible] babies to diphtheria and scarlet fever forms one of the most impor [illegible] mples of passive immunity acquired by inheritance. This depends on the passage of antibodies from the mother to the offsprings through the placental circulation and in some cases, through milk.

By artificial means:—

BACTERIOPHAGE THERAPY:—Described later.

SERUM THERAPY:—This may be carried out in the following ways:—

(a) BY THE USE OF SERUM OF AN IMMUNISED ANIMAL.—Such antisera may be antitoxic and antibacterial.

(i) *Antitoxic sera* are those which act chiefly by neutralisation of exotoxins elaborated by the bacteria in the body. Some examples of antitoxic sera are anti-diphtheritic, anti-tetanic, anti-dysenteric, anti-gas-gangrene, anti-botulinus, anti-scarlatinal sera and anti-venin (for snake venoms).

(ii) *Antibacterial sera* are those which act chiefly by destruction of bacteria and thereby preventing the increase of endotoxins. The examples are anti-pneumococcal, anti-meningococcal, anti-streptococcal, anti-plague and anti-anthrax (Sclavo's) sera.

(b) BY THE USE OF CONVALESCENT SERUM.—This serum is obtained from an individual recently recovered from the infection. This has been tried for the following diseases, such as measles, acute anterior poliomyelitis and scarlatina.

The difference between active and passive immunity is summed up in the following table:—

	ACTIVE IMMUNITY		*PASSIVE IMMUNITY*
1.	Immune substances are produced by the cells of the individual himself.	1.	Immune substances are preformed or ready-made and are passively transferred.
2.	Duration of immunity is longer, for several years.	2.	Duration of immunity is temporary and short-lived.
3.	Obtained either by an actual infection or by the injection of vaccines.	3.	Obtained by the injection of sera from immunised animals or convalescent patients.
4.	After injection there is a period of negative, followed by a positive, phase.	4.	No negative phase after the
5.	Reactions after injection may be (a) local, (b) focal and (c) general.	5.	No reactions except those due to foreign protein.
6.	Used mostly as a prophylactic measure. Also used for the treatment of subacute and chronic diseases.	6.	Prophylactic use is very limited. Mostly used for curative purposses, particularly for the treatment of acute diseases.
7.	Not transmissible to the fœtus.	7.	Transmissible to the fœtus and nursling.

BACTERIOPHAGE

(THE TWORT—D'HERELLE PHENOMENON)

Introduction:—In the early days of Bacteriology, it was found that sometimes, degenerative changes occurred in bacterial cultures, as a result of which portions of the growth disappeared altogether. In those days, it was thought to be due to autolytic changes, causing disintegration of the bacterial bodies and considered to be a process of natural decay. For this reason, no scientific notice was taken of this. In 1915, *Twort* while working on the bacteriology of vaccine lymph, first described peculiar glassy transparent patches occurring in the white colonies of *Staphylococcus* on agar slopes. These were found to extend into the bacterial growth, increasing at the expense of the latter. He also found that similar effects could be produced on a fresh young colony by inoculating the latter from the material from such a glassy area and in this way, the change could be continually transmitted from culture to culture for an indefinite period. He also showed that the lytic principle was present and was active in the bacteria-free filtrate obtained by passing the culture through a porcelain filter. The material was very powerful, being active in 1 in 1,000,000 dilution. It could not be grown in any artificial medium but it acted best and grew on young cultures only. It had no effect, however, on dead cultures of bacteria. It was destroyed by heating it at 60°C. for one hour. Its action appeared to be specific, exerting the lytic effect typically only on specific homologous bacterial growth.

In 1917, *d'Herelle* recorded similar effects in the case of the *dysentery bacillus*. He took a young broth culture of dysentery bacillus, to which he added a few drops of a bacteria-free filtrate from a dysentery stool and to his surprise, found that the turbid culture became clear. When a little fluid from the lysed culture was transferred to another young culture of dysentery bacillus, the same effect was noticed. In this way, he carried on the transfers for many hundred times without any loss of activity, quite unlike a bacterial growth which usually deteriorates on repeated subcultures. From these observations, d'Herelle argued that the lytic agent was capable of multiplying like a bacterium and that, as it grew only in a young living bacterial culture, it was a living agent, too small to be detected, a filter-passing ultra-microspic organism, whch behaved as a parasite of the bacteria themselves. In fact, he regarded this lytic process as due to a virus of bacteria. As the virus destroys and digests the bacteria, he applied the term *Bacteriophage*. We sometimes call it by its abbreviation *phage*.

Properties of bacteriophage:—

(i) *Lytic effect:*—It acts on bacteria in such a way as to bring about their lysis in some phase of their growth. Under its influence, many of the organisms in the culture fail to complete [illegible] division, as a result of which long filamentous forms develop. Later [illegible] the cells swell up and the bacterial bodies disintegrate completely. S[illegible]nges produce the characteristic appearance on the colonies. This [illegible] demonstrated when a well developed young broth culture, inoculat[illegible] the lytic principle is incubated for 4 to 6 hours and then spread over a plate, when a very characteristic appearance is obtained. Figure 70 shows the film of growth which is beset

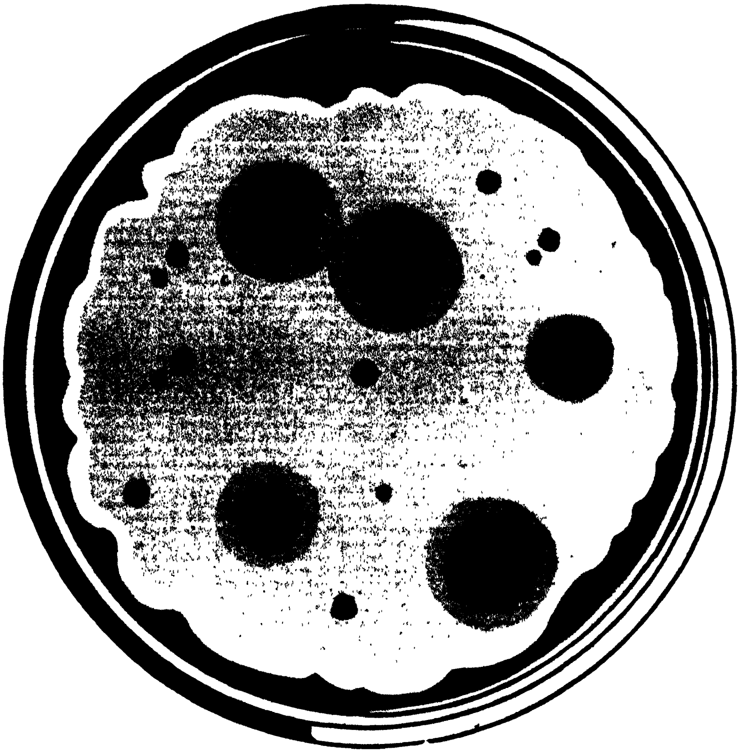


Fig. 70.

A film of growth showing the lytic action of bacteriophage; the circular areas represent the points of action of the phage.

with small circular areas, representing points of action of the lytic effect. Figure 71 shows the peculiar nibbled appearance of the colonies.

(ii) *Filterability:* It is filterable through a porcelain filter and the bacteria-free filtrate is equally active.

(iii) *Activity in high dilutions:* It is active in very high dilutions, even in 1 in 1,000,000,000.

(iv) *Power of propagation:* It can be propagated indefinitely without loss of activity, by repeated passage through young bacterial cultures.

(v) *Inability to grow in artificial media:* — It cannot be grown in any artificial medium like bacteria.

(vi) *Activity in cultures:* —It is most active only on young actively growing cultures. Dead bacteria or old cultures are little affected.

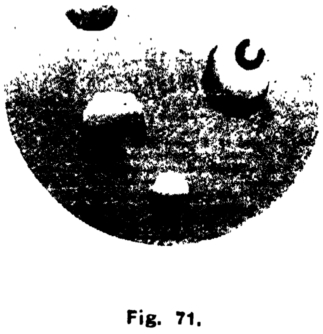


Fig. 71.

The peculiar nibbled appearance of colonies by the action of bacteriophage. Two normal colonies have also been shown.

(vii) *Specificity:*—It is usually specific; but may be slightly active on closely related species of bacteria. As a rule, one phage is specific for one kind of bacteria, e.g., cholera-phage is specific for cholera, dysentery-phage for dysentery and typhoid-phage for typhoid organisms, and so on.

(viii) *Adaptation:*—Bacteriophage may adapt itself to a particular organism and vice versa.

(ix) *Antigenic property:*—It is antigenic, i.e., antiserum may be produced by immunisation of a rabbit. This antiserum inhibits the activity of the lytic agent.

Sources:—Alimentary tract is the commonest source of bacteriophage. It is often found in the stool of healthy persons. The potent source is, however, from the stool of patients suffering from bacillary dysentery, cholera and typhoid fever.

Mode of preparation:—

1. A few drops of the stool are mixed with nutrient broth and incubated as usual for 18 hours.

2. It is then filtered through a Pasteur-Chamberland filter.

3. The filtrate contains the lytic principle and its presence can be demonstrated by inoculating a given culture of an organism with the filtrate.

4. After the bacteriophage is obtained, its action on a given organism can frequently be *exalted* by repeated passage from culture to culture till a certain level of activity is obtained.

Viability:

(i) Bacteriophage retains its power and activity for many years.

(ii) Faeces kept in sealed tubes for over a year may still be found to contain the bacteriophage unchanged.

(iii) It withstands drying for months.

(iv) It resists the action of antiseptics. In phenol, 1 in 100, it lives for 3 days.

(v) It persists for a long period in strong solutions of glycerin.

Nature of the bacteriophage:—There are two views about it *D'HERELLE* describes the bacteriophage as an autonomous, living, strictly parasitic organism, belonging to the group of filterable viruses, which utilises the bacterial substances in order to reproduce and cause dissolution of bacteria. According to him, bacteriophage is particulate in character, having an average dimension of 20 to 30 $\mu\mu$ (milli-micron) and is formed of a simple protoplasmic micella, a term used for an aggregate of molecules, ions, or both, in a colloidal solution. *BORDET*, on the other hand, is of opinion that it is of the nature of an enzyme, and therefore not a living virus. He thinks that it is a sort of an autolysis, due to the action of an enzyme derived from the bacteria and, like an enzyme, it is transmi

Methods of administration of bacteriophage:—Antiseptics or acids should not be administered during treatment with bacteriophage. As it works best in an alkaline medium, alkalis should be given freely, particularly a few minutes before its administration. It is best administered by the oral method and given in doses of 2 c.c. in a little water on an empty stomach and repeated 4 to 6 times a day. Usually these doses are enough to check an acute attack, but during the intervals, it is advisable to make the patient sip some more bacteriophage diluted with water. After recovery, it may be given once or twice a week. Ampoules showing any turbidity must not be used and once opened an ampoule must not be kept for further use. There are no contraindications to the use of the bacteriophage and it can be safely given to children in full doses.

Effect of bacteriophage therapy:—As it is not an antitoxin, mild doses of salines should be given to eliminate toxins already formed. Bacteriophage by destroying the infecting agent prevents the further progress of the disease and thus brings about a cure. It cannot heal the damage already done to the intestinal wall. Recent work of Morison and Rice (1933) in Assam has brought forward evidences to show that during cholera epidemics, early administration of 'phage,' i.e., within 48 hours, not only reduces the mortality but, of more importance, it seems to reduce the infectivity.

Bacteriophage and infection:—It has been suggested by the upholders of bacteriophage that the latter is definitely related with the cure of certain intestinal diseases, such as cholera, and also with the decline of its epidemic. When a person recovers from these diseases, it is due to the development of bacteriophage in his intestinal canal which causes lysis of the infecting organism and prevents further production of toxin. The death from such diseases is explained by the fact that the bacteriophage is absent in those cases. Once established, it continues to pass out along with the organisms in the dejecta and reaches the water supplies of the locality. The fresh victims ingest this water, infected with bacteriophage and the organism, but the latter fails to gain a foothold owing to the presence of the former. In this way, the disease gradually declines *pari passu* with the establishment of bacteriophage in the intestinal canal of the people of that locality. For these reasons, it has also been suggested that it is unwise to disinfect the water supplies, as by this method the natural means of prevention, i.e., bacteriophage, is lost. In this connection, it should be noted that Hankin in 1896 reported that filtered water taken from the Jumna and the Ganges rivers in India has a distinct bactericidal property. There are good reasons to believe that he was dealing with Bacteriophage phenomenon. At present it is very difficult to accept all the above statements *in toto* but recently it has been shown by many workers that bacteriophage certainly plays an important part in the cure and prevention of cholera.

CHAPTER XI

THEORIES OF IMMUNITY

We have so far described the various methods by which the resistance of an animal to infection can be increased. It now remains to explain in what way such an increased resistance is brought about. Every animal consists essentially of two elements, cells and fluids, and the development of immunity must be due to the action of either of them or both. Different observers have, from time to time, considered this question differently, some upholding the view that it is the result of cellular activity, whilst others consider the entire process to be the effect of the body fluids. Thus two rival theories were brought forward by two different schools, one led by Metchnikoff (Fig. 72) and the other by the great chemist, Ehrlich (Fig. 73).

Fig. 73.
Paul Ehrlich (1854-1915).

Fig. 72.
Elie Metchnikoff (1845-1916).

The former considered that the entire s of immunity was due to a
certain group of cells which were the phagocytes and thus
Metchnikoff's theory was referred to a llular theory or the theory of
phagocytosis. The greatest opponen views was Ehrlich who
believed that the cells had nothing to the development of immunity

which depended entirely on the property of the plasma, and on this belief, he based his humoral or side-chain theory. Although each school had its fervent supporters, none of them, however, could satisfactorily explain all the processes of immunity. Now-a-days, it is known that the real explanation is to be obtained by the combined action of the cells and plasma, both being interdependent on each other.

METCHNIKOFF'S THEORY OF PHAGOCYTOSIS
(Cellular hypothesis)

Metchnikoff believed that the leucocytes constitute the chief line of defence of the body against infection and intoxication, the bacteria or other invading organisms being destroyed and their products neutralised by enzymes derived from the leucocytes. This destruction of bacteria is brought about by chemical or enzymatic substances which he termed *cytases*, two of which have been distinguished. The one, derived from the polymorphonuclear leucocytes and mainly used for the destruction of bacteria, particularly the pyogenic organisms, is known as the *microcytase*. The other, derived from the large hyaline and other mononuclear leucocytes of the reticulo-endothelial system and used mainly for the digestion of cell bodies and elements of animal origin, is known as the *macrocytase*. According to Metchnikoff, the various kinds of ferments and enzymes are found chiefly inside the bodies of these cells but are also liberated in the plasma and serum, either by a process of excretion or by the disintegration of the cells. The production of various antibodies, such as antitoxins, agglutinins, precipitins, bacteriolysins, etc., is all explained by him through this theory.

Phagocytosis:—The word *phagocytosis* primarily means ingesting solid particles and eating them (*phagein*, to eat; *cyte*, cell). This process was oringinally and primarily nutritional in character, whereby the individual cell gained its food materials. Gradually, in the scale of development, as the structure of the animal body became increasingly complicated, and the process of nutrition was relegated to a different system, the phagocytic power of the cells came to be utilised for the purpose of engulfing and destroying harmful agents, such as bacteria. In this way, phagocytosis came to stand as a very important mechanism of defence.

Phagocytic cells:—Two types of cellular elements are concerned in this process and they are named by Metchnikoff as (1) microphage and (2) macrophage.

Microphages:— These are the polymorphonuclear neutrophilic leucocytes of the blood. They play the most important part and form the first line of defence in most bacterial infections. They are the principal fighting elements in all pyogenic infections.

Macrophages:—These are the large mononuclear leucocytes of the blood and the reticulo-endothelial cells of the liver, spleen, bone-marrow, lymph gland and other tissues. They act largely as scavengers, clearing away dead cells and other degenerated products and are thus the chief agents during the process of repair. In certain types of bacterial infection, particularly with Mycobacterium tuberculosis, they are the principal cellular agents concerned in the process of phagocytosis and M. tuberculosis can be demonstrated inside these large mononuclear cells. (Fig. 74).

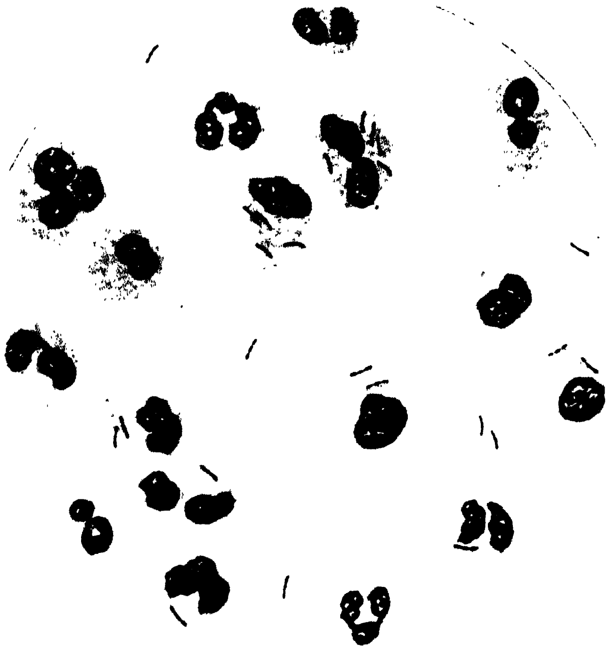


Fig. 74.

Phagocytosis of *Mycobacterium tuberculosis* by macrophages stained by Ziehl-Neelsen method. The red colour shows the acid-fast

Phenomenon of phagocytosis: - The process of phagocytosis is, more or less, a prolonged fight between the cells and the bacteria, and, for convenience, may be divided into three stages, although such a distinction is only arbitrary.

In the *FIRST STAGE*, the leucocytes mobilise at the site of the bacterial invasion and approach the bacteria to engulf them. (Fig. 75). When a pathogenic micro-organism is introduced into the tissues, there occurs an inflammatory reaction. The various phenomena that are seen during an inflammatory process are looked upon as nature's mechanism of defence which enables the tissues to fight and to protect themselves against any form of injury. It is a remarkable feature to note that before the commencement of the inflammatory process, there was hardly any cell outside the vessel, but within a short period, the perivascular spaces become loaded with the leucocytes which have emigrated from the blood vessels. Leucocytes must transmigrate or mobilise at the site of lesion for the proper phagocytosis to occur. But what causes this huge number of leucocytes to transmigrate? The various factors which are concern[illegible] the process are (i) hyperæmia of the part bringing in a large numbe[illegible] ocytes, (ii) increased permeability of the vessel wall, aided by the [illegible] movement of the leucocytes (diapedesis) and (iii) chemiotactic phen[illegible]

The term *chemiotaxis* is used to signify the attraction or repulsion of leucocytes by means of chemical substances elaborated by the bacteria, the nature of which is not yet definitely known. When the cells are attracted towards the seat of an infection, it is called a *positive chemiotaxis* and when they are repelled, a *negative chemiotaxis*. These chemiotactic substances have also a profound influence on the bone-marrow, where they may excite an increased production of leucocytes. In cases where a positive chemiotactic phenomenon is observed, it will be seen that not only are the leucocytes mobilised at the area of infection, but the increased demand is met by the bone-marrow by undergoing what is known as a *leucoblastic reaction* (Figs. 76 & 77).

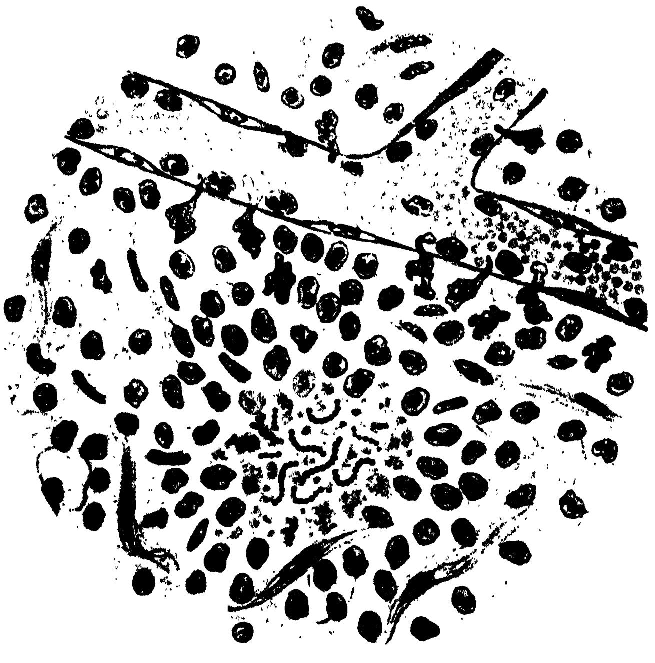


Fig. 75.

Mobilisation of leucocytes at an area of infection. The leucocytes are coming out of the blood vessels by their amœboid movements. Some of the organisms (streptococci) as well as the cells are dead in the process.

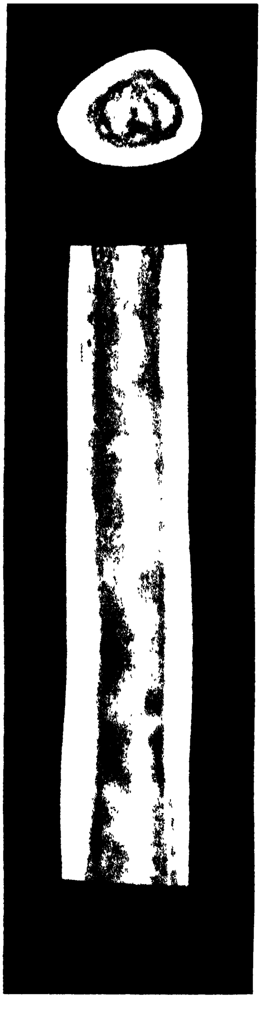


Fig. 76.

Longitudinal and transverse sections of the middle third of the femur showing leucoblastic reaction, in a case of lobar pneumonia with a high leucocytic response. Most of the normal yellow marrow has been transformed into the red formative marrow. The microscopic appearance of such a marrow is shown in Fig. 77 A.

Some of the mobilised leucocytes die during the contest between the cells and the bacteria and such dead cells are called *pus cells* (dead polymorphonuclear leucocytes). Accumulation of such pus cells has an important bearing on the prognosis of an wound infection, as *laudable pus* is recognised as a sign that all is going on well, whereas a thin watery discharge is a bad omen or a danger signal, signifying an infection with a virulent bacterium which has produced a negative chemiotaxis by repelling the leucocytes, and paralysing the blood-forming elements in the bone-marrow. Thus the accumulation of the most active phagocytic cells, the neutrophilic polymorphonuclear leucocytes, tends to prevent a localised infection from developing into a generalised one. The phenomenon of negative chemiotaxis is particularly seen in connection with the organisms of gas gangrene.

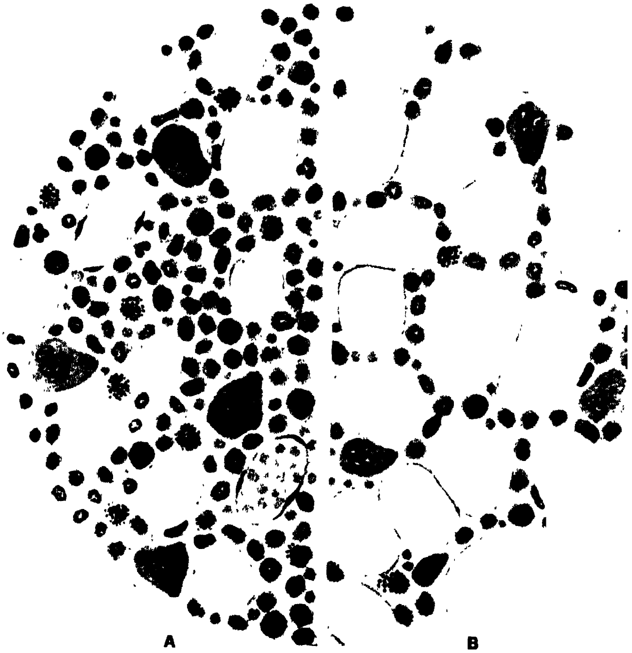


Fig. 77.

Microscopic appearance of the bone-marrow showing leucoblastic reaction (A) as compared with a normal yellow marrow (B). In B there is a large amount of fat (as shown by clear spaces) with very few cellular elements. In A the fat has been much reduced and its place has been taken up by blood-forming cellular elements, specially the white blood corpuscles.

In the *SECOND STAGE*, the leucocytes, after being aggregated at the site of infection, begin to devour the bacteria. The ingestion of bacteria by the leucocytes is effected by their amœboid movements. When the leucocytes approach the bacteria, they throw out pseudopodia, engulf and ultimately incorporate them within their own bodies (Fig. 78a, b, c). It was at one time thought that these phagocytes would devour only those bacteria which were dead and degenerating, but Metchnikoff proved beyond doubt, that they could also ingest living and virulent organisms.

In the *THIRD STAGE*, the ingested bacteria undergo intracellular lysis (Fig. 78 d, e). They are destroyed by the activity of the digestive ferments (cytases) of the phagocytes present inside the cells. Sometimes it happens that the organisms may remain [illegible] inside the cells for a long time without undergoing destruction, as [illegible] be seen with Mycobacterium tuberculosis, and even in some cases [illegible] actively proliferate within the cell as in Neisseria gonorrhœæ. [illegible] certain circumstances, these phagocytes, by carrying living and virulent organisms, may be dangerous to the animal, as it affords a chance of wider dissemination.

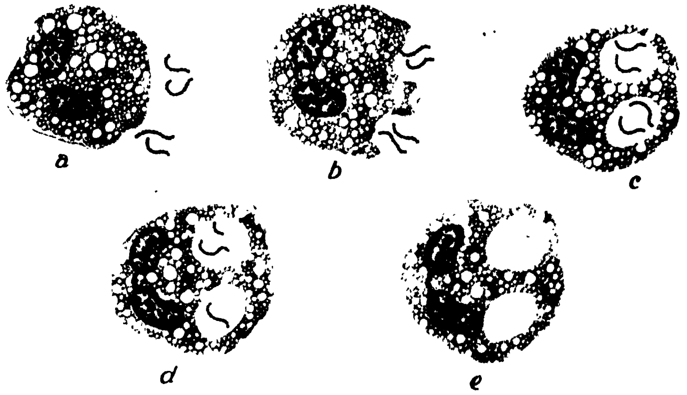


Fig. 78.
Phenomenon of phagocytosis.

Influence of opsonins in phagocytosis:—As will be seen later, the presence of opsonins is absolutely essential for the proper phagocytosis to occur. This substance is present in the normal serum and, in the process of phagocytosis, they play a great rôle in making the bacteria *palatable* to the cells. This they do by altering the bacteria in such a way as to render them more suitable for ingestion by the cells. When the cells (phagocytes) are deprived of its assistance, they are unable to ingest the bacteria. To such a substance *which prepares a feast*, the term opsonin has been applied by Wright and Douglas (Gr. *opsono*, to cater for).

EHRLICH'S SIDE-CHAIN THEORY

This is a humoral theory which refers to the destruction of the invading bacteria and their toxic products by the chemical action of substances in the body fluids. It is an application of Ehrlich's views regarding the normal nutrition of the body cells to which he attributes a hypothetical chemical structure. He considers that every cell in the body consists of an aggregation of molecules, each of which again consists of a central atom-group and a large number of appendages or side-chains, exactly similar to the lateral chains of many chemical molecules. To such imaginary side-chains, he gave the name *receptors*. All food materials which are brought to the cells for the purpose of nutrition are supposed to attach themselves by means of similar side-chains, which are called the haptophores, first to these receptors, before they can be utilised and incorporated into the essential cell substance. The receptors serve as the contact points or intermediaries between the body cells and the nutrient particles and without them, no combination can take place. Thus the vital activities of the living cells are the result of chemical interaction between the side-chains and the food elements.

He also conceived that these side-chains, although primarily taken up by the food particles under normal physiological conditions, have also combining affinities for substances of the class to which antigens belong, e.g., toxins, bacteria and their products, foreign cells, etc. Therefore, when both the food particles and antigenic substances reach the body cells, some of the receptors of the latter attach themselves to the haptophores of the food elements, while the others combine with those of the antigen. Those which are anchored to the latter are diverted from their normal physiological functions and the body cells are thus deprived of them. Such a loss of side-chains at once stimulates the cells to throw out more similar side-chains to make good the loss. But if the supply of the antigen continues, *pari passu*, the loss of side-chains also continues and the result is the continuous activity of the cells to produce receptors to meet the increasing demand. Nature is generous and this make-good process is carried to excess, as a result of which a similar type of receptors is thrown out of the cell body in much larger numbers than what is actually needed to compensate for the loss. These superfluous receptors which are thrown out of the cells and which freely circulate in the blood, constitute, according to Ehrlich, the various kinds of *antibodies*.

(antibodies) are

first order—These include

2. Receptors
(i) agglutinins and

3. Receptors of the third order—These include amboceptors or immune-bodies, such as, (i) various lytic bodies bacteriolysins, hæmolysins, cytolysins; (ii) complement-fixing bodies and (iii) opsonic immune bodies.

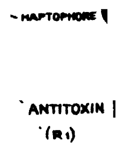


Fig. 79.
Receptors of the first order (R1) represented graphically. Showing a single combining arm H which unites with the haptophore group of the toxin molecule.

Receptors of the first order:—To this type belong the antitoxins. These possess a single unsatisfied valency or side-chain (Fig. 79 a) which combines with the haptophore group of the corresponding antigen. The free receptors, present in the circulating blood, block the haptophore groups of the toxin molecules in the blood (Fig. 79 b) and prevent their combination with the receptors still attached to the cells and thus the cells are protected from the poisonous action of the toxin.

Receptors of the second order (Fig. 80):—To this type belong the agglutinins and precipitins. They consist of one anchoring group (haptophore) which fixes the antigen and the other active or zymophore group, which leads to some physical change in the fixed antigen. In this way, phenomena of agglutination of bacteria and precipitation of antigens are explained.

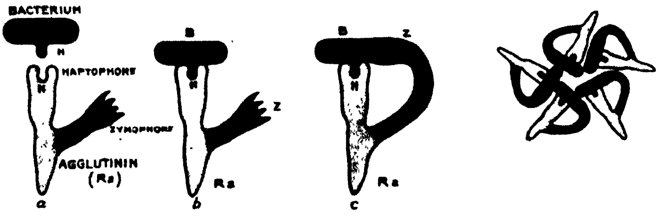


Fig. 80.

Receptors of the second order (R2) represented graphically. Showing a combining arm H which unites with the bacterial antigen and another side-arm Z which brings about some alteration in the bacterial antigen to produce the clumping: a agglutinin (R2) and bacterium (B) are separate; b, union of R2 and B, c, shows the action of zymophoric arm after the antigen has been fixed with its specific antibody; d, shows the clumping and at this stage the presence of an electrolyte is necessary.

Receptors of the third order (Fig. 81): To this type belong the various lytic bodies. They consist of two anchoring groups (haptophores) one on each side (Fig. 81 a) and hence called *amboceptor* (I seize both). One group anchors the corresponding antigen and the other group combines with a ferment-like substance, called complement (Fig. 81 b). Amboceptor is capable of assimilating the corresponding or specific antigen only with the help of the complement.

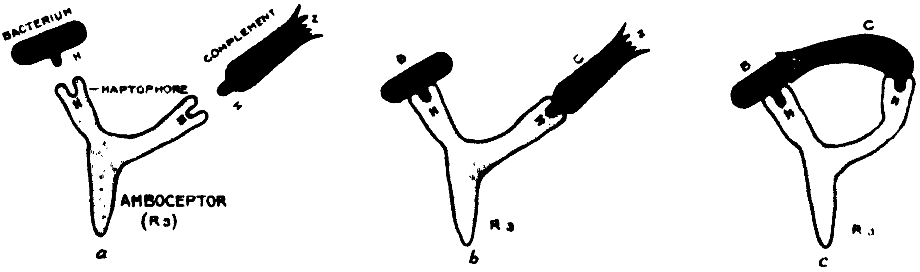


Fig. 81.

Receptors of the third order (R3) represented graphically. a, the amboceptor with its two haptophore groups, complement and bacterium, b, the complement and the bacterium have united with the amboceptor, c, shows the action of complement on the antigen-antibody complex

Genesis of Antibodies (immune substances) on the Basis of Ehrlich's Side-chain Theory.

(Type of immune substance represented is antitoxin.)

Ehrlich believes that a toxin molecule consists of two separate atom-groups. One, the *haptophore* or the *anchoring* group, which unites the toxin molecule with the receptor of the cell protoplasm; and the other, the *toxophore* or the *poison* group which exerts its deleterious action on the cell. The toxophore group, however, cannot inflict injury to the cell, until and unless the toxin molecule is anchored to the receptor of the cell protoplasm through its haptophore group.

If a dose of toxin is introduced into the blood, the haptophore groups of the toxin molecules combine with the corresponding receptors of the cells and interfere with the normal physiological activities of the cell protoplasm which is thus damaged. If the number of toxin molecules united to the cell is very large, most of its receptors are taken up by the former, interfering seriously with the process of nutrition and assimilation and the cell may thus be killed. But provided the dose of toxin is not too large, the protoplasm recovers and, according to Ehrlich, this recovery is due to a regeneration of the side-chains or receptors in place of those which have been put out of action. These receptors are not simply reproduced in the same quantity in which they are lost but are reproduced in excess of the simple physiological needs of the cell. Continuous and increasing doses of toxin will lead to an excessive production of these particular receptors, until the cells become overstocked with them and cast them off to circulate freely in the blood. These freely circulating receptors represent the corresponding antibodies (antitoxins) and form the reserve resources of the immunised animal. They are able to combine with the poison and neutralise it, before it can reach the sensitive cell to produce its deleterious action.

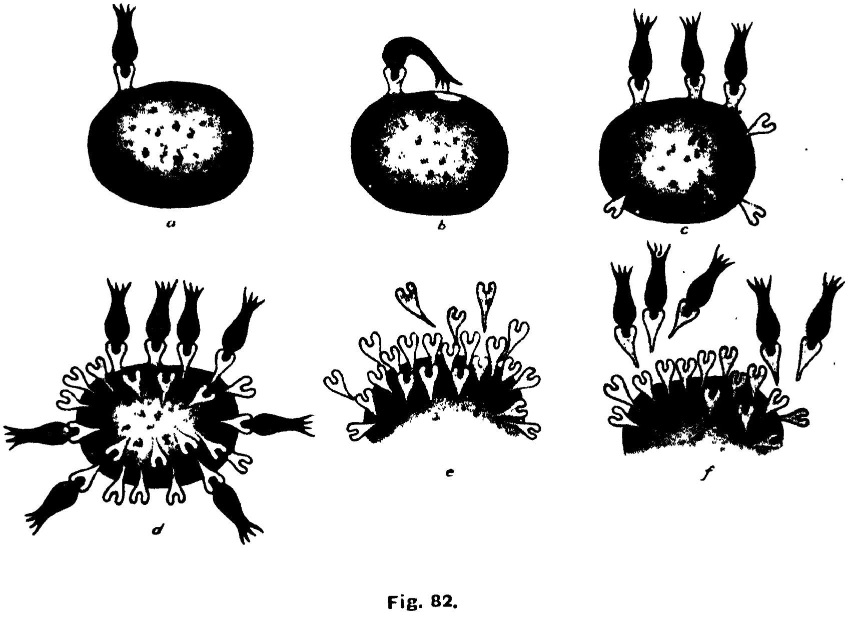


Fig. 82.
Genesis of antitoxin.

The excessive regeneration or over-production of side-chains (receptors or antibodies) under the influence of repeated stimulation can be explained by a physiological law enunciated by Weigert. Regenerative process of the body cells does not stop at simply replacing the lost element but, according to Weigert's hypothesis, usually leads to over-compensation. This is met with in various pathological conditions of regeneration and repair. Thus the process of antibody formation representing antitoxin may be summed up and represented graphically in Figure 82.

First stage—Fixation of toxin or the poison and its union with the cell (Fig. 82 a). After the union, the toxophore is damaging the cell protoplasm (Fig. 82 b) as is shown by the clearing of area.

Second stage—Formation of new receptors (Fig. 82 c).

Third stage—Over-production of receptors (Fig. 82 d).

Fourth stage—Setting free of receptors produced in excess (Fig. 82 e).

Fifth stage—Neutralisation of toxin by antitoxin (free receptors) in the blood (Fig. 82 f).

RETICULO-ENDOTHELIAL SYSTEM AND ITS RÔLE IN THE PROBLEMS OF IMMUNITY

This forms a special group of cells which abound in the liver, spleen, lymphatic glands and bone-marrow and are also present in varying numbers in other parts of the body. They have a common morphology and function and are called by Aschoff (1924) and Kiyono (1914) as the reticulo-endothelial cells, and by others as histiocytes or clasmatocytes. One of the many functions which is common to all of them is their power of phagocytosis and of engulfiing and storing of pigments or of any foreign particulate matter introduced into the system, either artificially, as by vital staining, or naturally, as in the process of infection. They are derived from those cells of the primitive mesenchyme which goes to form the blood vascular system in the embryo and are distributed widely throughout the animal body and are not confined to any single organ. Their distribution may be summarised as follows:—

Cells of the reticulo-endothelial system
(Histiocytes)

FIXED MACROPHAGES (SESSILE HISTIOCYTES)	*WANDERING MACROPHAGES (WANDERING HISTIOCYTES)*
(i) Endothelial cells of the liver capillaries (the littoral cells of Kupffer)	*(i) Tissue macrophages (Tissue histiocytes or the wandering mononuclear cells)*
(ii) Cells of the splenic pulp	*(ii) Monocytes of blood or blood macrophages (Blood histiocytes)*
(iii) Mononuclear cells of the sinuses of the lymphatic glands	
(iv) Branched reticular cells of the bone-marrow	

This system is endowed with many important functions such as
(a) Storage and metabolism of iron.
(b) Metabolic functions, particularly cholesterol metabolism.
(c) Blood formation, specially in the bone-marrow.
(d) Blood destruction, particularly in the spleen.
(e) Formation of bile from blood pigment.
(f) *Phagocytosis of bacteria and foreign particles in the blood.*
(g) *Immunity reactions and defensive mechanism, by (i) phagocytosis and (ii) formation of immune bodies.*

The last two are the most important properties which are specially concerned in the problems of immunity.

In this connection it should be noted that by increasing its functional capacity in one direction, other functions may be more or less put out of gear. This is according to the general principle that whenever there is overactivity of one function, other activities of the organ are diminished or are in abeyance. When the activities of the reticulo-endothelial system are diverted in this way, the process is known as "blockading" or " blocking," a phenomenon commonly employed in experimental pathology to throw this system out of action. The method consists in introducing into the system foreign insoluble materials, such as carbon particles (Indian ink), dye stuffs or heavy metals, whereby the cells are forced to be actively engaged in dealing with them. At this stage of active phagocytosis of foreign particles by all the available macrophages, there is depression of other activities of the system. Lastly it should be remembered that the normal excitability of the reticulo-endothelial system is very greatly reduced by defects in diet and particularly by a lack of vitamins.

RELATION TO IMMUNITY

The cells of the R. E. system, as already mentioned, are endowed with the power of engulfing any foreign particulate matter introduced into the system by any means and storing and ultimately disposing them of. They are also attributed with responding to foreign materials, not only by phagocytosis but also by active multiplication. When the stimulus is over and their function is no longer required, they diminish in number and once more get back to their respective sites. But their heightened power to respond to any fresh stimulus of a similar nature, by immediate proliferation and increased phagocytic activity, remains more or less as a permanent feature and it is on this that the immunity of the animal depends.

Immunity reactions and defensive mechanism:—The activities of the R. E. system in the problems of local and general defence may be considered under two heads:—(i) as a source of macrophages and (ii) as the site of antibody formation.

(i) **Source of macrophages:**—The macrophages belonging to the **reticulo-endothelial system**, according to Metchnikoff, are the all important defensive elements. They are endowed with the power of engulfing and ultimately destroying the invading agents by a ferment, called macrocytase. In the various infective processes, natural or induced, these cells are not only rapidly mobilised but there results an accelerated proliferation, both local and general, the resistance of the individual depending on the rate and the degree with which they can multiply.

(ii) **Site of antibody formation:**—The most important function of these cell-elements appears to be the manufacture of antibodies and it has been definitely proved by modern scientific works, that all the antibodies which are detected in the serum of animals are primarily elaborated by these cells which subsequently pass them into the plasma and lymph. That antibodies are formed by the cells of the reticulo-endothelial system has been definitely proved by "blockading" the cells or removing the spleen (splenectomy), the largest depôt of these cells. By such means, it has been shown by many workers that all the various factors in immunity, viz., agglutinins, precipitins, bacteriolysins, cytolysins, hæmolysins, etc., are either not formed at all or formed in very much less amount than in a normal animal. Such a splenectomised or blockaded animal will often succumb to an infection which, in a normal one, would not be lethal. Of course, this state of lowered general resistance will not last for ever, as the reticulo-endothelial cells in other organs will undergo compensatory hyperplasia and make up for the loss sustained by the removal of the spleen or blockading.

There are some diseases which, in their course, produce varying degrees of blockading of the reticulo-endothelial systems, thus lowering the resistance of the patients to infection. Every student and practitioner in our country will find an excellent example of such a condition in the disease known as Kala-azar. In this illness, every available reticulo-endothelial cell in the liver, spleen, bone-marrow and other parts of the body is found to engulf and hold within the cytoplasm enormous numbers of the parasites of the disease, viz. the Leishmania donovani. This serves as a perfect blockading. In their pre-occupation to deal with the parasites, the cells fail to discharge their other functions, specially those concerned with immunity reactions and the result of such a condition is the development of pneumonia, gangrenous stomatitis, ulcerative colitis and a host of other serious infective processes to which a Kala-azar patient is constantly liable.

CHAPTER XII

OPSONINS

Definition:—Wright and Douglas (1903) demonstrated that in the presence of normal serum, bacteria were ingested by leucocytes, but when the serum was heated to 55°C., the phagocytic power of the leucocytes disappeared. This showed that some substance, present in the normal serum, was destroyed by heating and the absence of which retarded the process of phagocytosis. To such a thermolabile substance in the normal serum, they gave the name of opsonins (*Gr. opsono*, to cater for).

Distribution of opsonins:—They are mainly present in the blood. Extracts of various organs show absence of any opsonic action. Cerebro-spinal fluid, aqueous humour, milk and seminal fluid are found to contain none. It has also been observed that the opsonic action of the fluid of subcutaneous tissues is much weaker than that of the blood. The opsonic activity of the foetal blood'is less than that of the maternal. Immediately after birth, the opsonic content of the new born decreases for some months, then rises gradually and reaches the normal level by the end of the second year.

Opsonins in immune sera:—The phagocytosis-promoting action of a serum, developed during the process of immunisation, is not due to the increase of the normal opsonins, but to an antibody similar to opsonins and remarkable for its two characteristic properties.

(i) It is specific towards the organism used in the process.

(ii) It is thermo-stable i.e. like all anti-bodies, it is resistant to heating at 55°C.

Thus, the increased opsonic action of an anti-bacterial serum (such as anti-pneumococcal serum) is due to a specific thermostable antibody (immune opsonin). Such an antibody presents two types of action.

1. When the opsonic effect is produced without the inter-action of the complement, it is termed bacteriotropins (Neufeld), i.e., the action is a direct one on the organism and thus it may be included in Ehrlich's receptors of the second order.

2. When the opsonic effect is produced by the interaction of the complement and the antibody, it is termed the opsonic immune body or immune opsonin and may be included in Ehrlich's receptors of the third order.

Mode of action of the opsonic substances:—The substances which promote phagocytosis, whether they be of a thermo-labile non-specific type (normal opsonins) or thermostable specific type (immune opsonins or bacteriotropins), have a direct action on the bacteria. The opsonic agent produces some physical change in the bacterial envelope and such alteration appears to be accompanied by an increased adhesiveness. Another peculiarity of this change is that after the organism has been opsonised, the effect of the opsonin cannot be abolished by heating.

Opsonic immune body and its antigen:—The antigen which increases opsonic action is the nucleoprotein content in the bodies of the bacteria. In case of the flagellated organisms, it is the somatic antigen (O antigen) or endoplasm that gives rise to the opsonic antibody.

OPSONIC INDEX

This term was introduced by Wright to indicate the relation between the phagocytic action of a given number of leucocytes in a particular serum and that of a similar number of leucocytes in a normal serum. It is a measure of the opsonic power of a serum and may be expressed as follows:

$$\text{Opsonic index} = \frac{\text{Average number of bacteria ingested per leucocyte in the presence of the patient's serum.}}{\text{Average number of bacteria ingested per leucocyte in the presence of a normal serum.}}$$

The normal index for a healthy person is usually 1, but it may vary from 0·8 to 1·2.

Technique for the determination of the opsonic index:—

MATERIALS REQUIRED:

1. A suspension of organisms.
2. Patient's serum.
3. Control serum (normal serum).
4. Blood cells (leucocytes).
5. A teated capillary pipette.
6. Staining reagents, either Leishman's or Ziehl Neelsen's stain; the latter is useful particularly in the case of M. tuberculosis.

Preparation of a suspension of organisms:—The bacterial emulsion is prepared in such a way as to obtain a suspension which is just opalescent to the naked eye; this requ'res a little practice. Any clumps that might be present in the mixture should be removed, as it is essential that the bacteria should remain isolated from one another. A few loopfuls of a twenty-four hours' agar culture are taken and rubbed up thoroughly in a few cubic centimetres of normal saline in a watch glass. A homogeneous suspension is prepared and any clumps present may be removed by centrifugalisation. With highly pathogenic organisms, it is desirable to use a culture which has been killed either by heat or by the addition of formalin. The latter is then eliminated by centrifuging and washing in saline. It is very difficult to prepare an emulsion of tubercle bacilli for which a special technique is required.

Preparation of leucocytes:—Five to six cubic centimetres of a 1·5 per cent. sodium citrate solution in normal saline are taken in a centrifuge tube and about 10 to 15 drops of blood are allowed to flow from the observer's own finger into the solution. The mixture is then centrifuged at a low speed and when the corpuscles are separated, the supernatant fluid is removed. Fresh saline is added to the deposit which is again centrifuged. The deposit consists of a thin greyish pellicle, which contains chiefly the leucocytes and is removed by a fine pipette. It is then thoroughly mixed with normal saline. Such a preparation will suffice for about a dozen tests. It is of course not possible to obtain leucocytes without the admixture of red cells.

Preparation of the sera:—These are obtained in the usual way, by receiving the blood in a test tube or a glass capsule or a Behring's venule (see under agglutination reaction). After clotting has occurred, the serum is pipetted off. These sera should always be freshly prepared.

METHOD:—Equal quantities of a preparation of leucocytes, patient's serum and a suspension of the organisms in question, are measured off with the capillary pipette, as shown in Figure 83. They are then thoroughly mixed in a small tube and incubated for 20 minutes at body temperature (37 °C.). After incubation, film preparations are made from the mixture and suitably stained according to the particular organism to be tested. They are then examined microscopically and the average number of bacteria phagocytosed by one leucocyte is estimated. This indicates the phagocytic index of the patient's serum. A similar experiment is performed with normal serum and its phagocytic index is again estimated. The opsonic index is calculated by dividing the phagocytic index of the patient's serum by that of the normal serum.

Fig. 83.

Method of using a capillary pipette for measuring equal volumes of bacteria, serum, and leucocytes as in the determination of the opsonic index. The clear areas represent the air-spaces in between the materials.

Uses of opsonic index:—

(1) For diagnosis, e.g., in tuberculosis.

(2) As a measure of immunity.

Effect of an injection of a vaccine on the opsonin content of the patient's serum:—

The immediate result of such an injection is the lowering of the opsonin content of the serum and this is due to the linking-up of the available opsonins with the bacteria. This condi[illegible] spoken of as the negative phase. During this period, the patient [illegible] feel well and there occurs an aggravation of the local lesion that [illegible] present, such as the appearance of fresh crops of boils in furuncu[illegible] This negative phase usually lasts for 2 to 3 days, but may vary from a few hours to a week or 10 days, depending on the dose. Thus, its duration may be increased by a larger dose or reduced and completely eliminated by using a smaller dose. After this negative phase, there is a fresh supply of opsonin, owing to the stimulus provided by the injection and hence the opsonin content of the serum rises, producing a condition known as the positive phase. After reaching the maximum, the opsonic index oscillates, then comes to a stage of equilibrium, after which it declines gradually to or below the original normal level. Thus the effect of a vaccine injection on the opsonic index may be divided into 2 phases:—

(1) *Negative phase*—Average duration is about 2 to 3 days, which however varies with the size of the dose. Injection should not be repeated during this period.

(2) *Positive phase* which may be divided into 3 periods :

(a) Stage of fastigium.

(b) Stage of equilibrium.

(c) Stage of decline.

All these taken together form what is known as the opsonic cycle and may be represented graphically, as in Fig. 84 A.

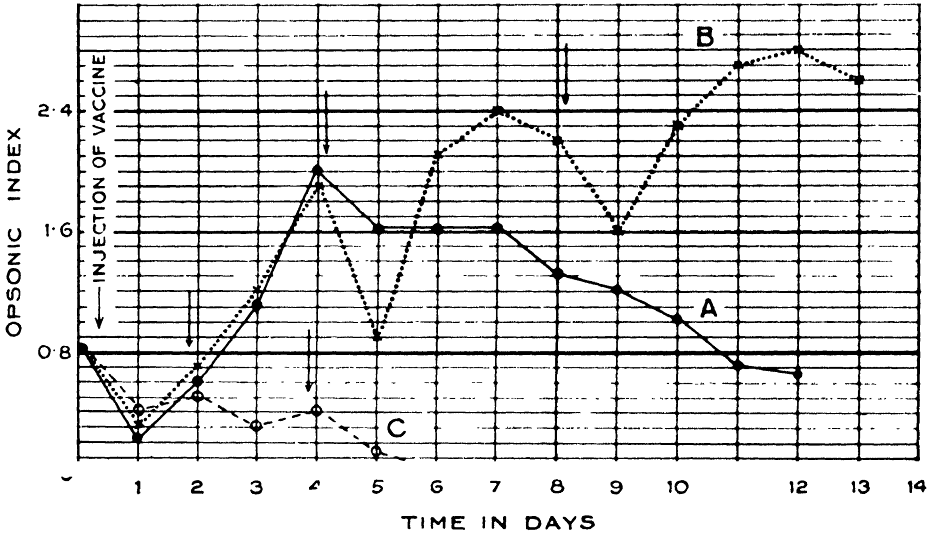


Fig. 84.

Effect of vaccine injections on opsonic index

A Normal opsonic cycle.

B—Gradual rise of opsonic index, produced as a result of repeated injections of vaccines during the positive phase.

C -Decline of the opsonic index, as a result of repeating the injection at short intervals i.e., during the negative phase.

Consequently, to obtain the best results with vaccine therapy, it is advisable to repeat the second injection towards the end of the fastigium. The doses should be so adjusted as to avoid the negative phase altogether or to have it as short as possible. The injections should be repeated during the positive phase which lasts for 4 to 7 days and in this way, active immunity is built up. In this connection, it should be noted that by successive injections during the positive phase, the immunity reaches to a stage beyond which it cannot be further increased. The effects, on the opsonic index, of giving injections in the negative and positive phases are shown in Figure 84 B & C.

COMMON BACTERIAL VACCINES

A vaccine consists of a suspension of killed or living bacteria in normal saline, and is used as an injection into the tissues of an animal or patient, to create a high degree of immunity against infection by the same organisms. This is one of the methods of inducing artificially active general immunity by stimulating the production of bactericidal substances in the body.

PREPARATION OF AUTOGENOUS VACCINES

STAGES: -

1. Cultivation of the infective material on appropriate culture media.
2. Isolation of the organism in pure culture on suitable solid media.
3. Emulsification in normal saline. This must be uniform and homogeneous.
4. Sterilisation in water-bath at 55° to 60°C. for ½ to 1 hour.
5. Standardisation, i.e. determination of the approximate number of the organisms in a given quantity.
6. Controlling, i.e. testing for sterility.

Before cultivating, it is advisable to examine a stained smear preparation of the infective material in order to get an idea of the probable organisms present in it and also to determine the predominance of any particular type. Primary cultures are then made on the surface of suitable solid media under appropriate conditions; usually agar or blood agar is most commonly employed. After incubation, the selected colonies of the required organisms are fished out and subcultured on slopes or plates. In the case of a mixture of organisms, each individual bacterium should be subcultured separately. About 1 to 2 c.c. of carbol-saline (0·25 to 0·5 per cent. phenol in sterile normal saline) are poured into the culture tubes which are then gently shaken, until the growth becomes detached from the medium. This process may be facilitated by scraping the colonies from the surface of the medium with a sterilised platinum loop. Emulsion of each organism is collected into a sterile test-tube, and examined to see whether it is homogeneous and uniform. If clumps are present, these must be disintegrated by further shaking, preferably in the presence of sterile glass beads. They may also be deposited, either by centrifuging at a low speed or keeping the emulsion at rest for an hour, to allow large masses to fall at the bottom. The supernatant fluid may be decanted or pipetted off into a fresh tube. This is then sterilised in a water-bath (vaccine bath) at 55° to 60°C. for half to one hour. This low temperature kills the bacteria, but does not affect the antigenic property which is lost easily at higher temperatures.

After heating, the sterility is tested by transferring five or six loopfuls of the emulsion into a tube of broth or other suitable *fluid* media. This is an important technical point which should be observed, since, owing to the presence of phenol, it is useless to attempt a culture on a solid medium. Counting of bacteria may be performed preferably after the sterilisation of the emulsion. During the various stages of the preparation of a vaccine, it is necessary to employ utmost precautions to guard against accidental contaminations. To ensure this, the various operations should be performed in the vicinity of the flame. A sterilised towel soaked in 1 in 20 carbolic lotion may be placed on the working table over which materials are kept and handled.

Enumeration of the organisms in the emulsion: In making vaccines, it is advisable to know roughly the total number of bacterial cells present in the emulsion. Counting of bacteria in a vaccine emulsion may be done in a variety of ways, such as:—

(1) *Hæmacytometer method*—The organisms are counted like red blood corpuscles.

(2) *Wright's method*—Equal volumes of whole blood (whose R.B.C. count is known) and the bacterial emulsion are mixed and a film prepared and stained with Leishman's stain. The total numbers of red cells and bacteria in a definite number of fields are counted separately. The number of organisms per c.c. is obtained by comparing the number of bacteria with the number of corpuscles. The method is very laborious and the results are somewhat unsatisfactory.

(3) *Comparison with standard opacity tubes*—This method is now usually employed by comparing the opacity of a sample of vaccine with that of a series of opacity tubes (Brown's), which may be purchased from Messrs. Burroughs Welcome and Company. The apparatus consists of a set of ten standard tubes, containing various dilutions of barium sulphate suspended in 1 per cent. sodium citrate solution. These should be thoroughly shaken before use. Different organisms in suspensions of the same concentration have different degrees of opacity and it is necessary to have at hand standards of different organisms. A leaflet (see table on p. 147) is supplied with the apparatus which shows the relation of opacity to the numerical equivalents of various bacteria in millions per cubic centimetre, estimated by means of the haemacytometer method. Tubes of the same diameter and thickness are supplied, in which a sample of vaccine may be placed and compared with the opacity tubes. The matching is done by reading printed matters through suspensions, and the number of organisms per c.c. can be obtained from the attached table. For example, in finding out the strength of an emulsion of E. typhi, if it is found to match with opacity tube No. 5, then the number of organisms in that particular emulsion is 2289 millions per c.c.

Cunningham and Timothy's modification of Brown's opacity tubes gives better results, because the suspension is made in a jelly which does not require any shaking. It also does not alter its colour in the tropical climate. **But** ***it should always be kept in a refrigerator.***

Table showing the relation of opacity to the numerical equivalent of various bacteria, estimated by means of the hæmacytometer method, in millions per c. c.

Opacity Tube No.	Staphylococcus pyogenes aureus.	Staphylococcus pyogenes albus.	Streptococcus (non-hæmolytic).	Streptococcus (hæmolytic).	Escherichia coli (B. coli communis).	Eberthella typhi (B. typhosus).	Salmonella paratyphi (B. paratyphosus A).	Salmonella schottmulleri (B. paratyphosus B).	Eberthella dysenteriæ (B. dysenteriæ, Shiga).	Eberthella paradysenteriæ (B. dysenteriæ, Flexner).	Vibrio comma (V. choleræ).	Pseudomonas aeruginosa (B. pyocyaneus).	Klebsiella pneumoniæ (B. pneumoniæ, Friedländer).	D. pneumoniæ (Pneumococcus).	Neisseria catarrhalis (M. catarrhalis) on agar-agar.	Neisseria catarrhalis (M. catarrhalis) on agar-serum.	Hæmophilus influenzæ (B. influenzæ, Pfeiffer).	Neisseria gonorrhœæ (Gonococcus).	Neisseria meningitidis (Meningococcus).	Brucella melitensis (M. melitensis).	Corynebacterium acne (B. acnes).
10	3789	3390	3313	3043	3787	4577	4222	4171	4629	4494	10926	5481	4032	7053	3611	3651	11396	3578	5084	7684	5368
9	3410	3051	2982	2739	3408	4119	3800	3754	4166	4045	9833	4933	3629	6348	3250	3286	10256	3220	4576	6916	4831
8	3031	2712	2650	2434	3030	3662	3378	3337	3703	3595	8741	4385	3226	5642	2888	2921	9117	2862	4067	6147	4294
7	2652	2373	2319	2130	2651	3204	2955	2920	3240	3146	7648	3837	2822	4937	2528	2556	7977	2505	3559	5379	3758
6	2273	2034	1988	1826	2272	2746	2533	2503	2777	2696	6556	3289	2419	4232	2167	2191	6838	2147	3050	4610	3221
5	1895	1695	1657	1522	1894	2289	2111	2086	2315	2247	5463	2741	2016	3527	1806	1826	5698	1789	2542	3842	2684
4	1516	1356	1325	1217	1515	1831	1689	1668	1852	1798	4370	2192	1613	2821	1444	1460	4558	1431	2034	3074	2147
3	1137	1017	994	913	1136	1373	1267	1251	1389	1348	3278	1644	1210	2116	1083	1095	3419	1073	1525	2305	1610
2	758	678	663	609	757	915	844	834	926	899	2185	1096	806	1411	722	730	2279	716	1017	1537	1074
1	379	339	331	304	379	458	422	417	463	499	1093	548	403	705	361	365	1140	358	508	768	537

Distribution of vaccines:—Vaccines may be put aseptically in 1 c.c. glass ampoules or rubber-capped bottles.

(a) Appropriate doses are measured and the contents put into the ampoules by means of a sterilised glass syringe. These may then be mixed with carbol-saline to make them up to 1 c.c. each, or any other quantity as desired. When charged, the ampoules are sealed. These are mainly used for supplying a series of graduated doses and usually 6 to 10 ampoules are supplied. Each ampoule is intended for a single dose. Before use, it should be vigorously shaken.

(b) Rubber-capped vaccine bottles of 10 to 20 c.c. capacity are also used. The bottle is sterilised in an autoclave and the rubber-cap, by boiling in 5 per cent. carbolic acid for 20 minutes to half an hour. After charging the bottle with the vaccine, the rubber-cap is fitted on to it and sealed with paraffin wax. The bottle is then properly labelled and is ready for use.

After preparation, the vaccines, if not used immediately, should be kept in a cold chamber, as the antigenic potency of such a prepared vaccine cannot be maintained for more than 6 months, and even very much less in the tropics.

Dosage of vaccines:—No definite rules can be laid down for the dosage of vaccines. It depends on many factors, such as:

(i) The purpose for which they are used.
(ii) The type of the case.
(iii) The general condition of the patient.
(iv) Age of the patient.
(v) The clinical judgment of the medical attendant.

In general, it may be said that it is better to begin with a small dose and the successive doses are regulated by watching the reaction produced. It is desirable to avoid big reactions and to aim at slight local and general reactions. Administration of large doses will produce definite harm to the patient, as it tends to depress rather than exalt, the patient's resistance.

For treatment, the following table will give some indication as to the initial dosage:—

Staphylococcus	25	millions.
Streptococcus hæmolyticus	2	,,
Streptococcus non-hæmolyticus	5	,,
Diplococcus pneumoniæ	5	,,
Klebsiella pneumoniæ (Friedländer's pneumobacillus)	10	,,
Hæmophilus influenzæ	10	,,
Neisseria catarrhalis	25	,,
Neisseria gonorrhœæ	5	,,
Diphtheroid bacilli	20	,,
Eberthella typhi (B. typhosus)	5	,,
Eberthella para-dysenteriæ (B. dysenteriæ, Flexner)	2	,,
Eberthella dysenteriæ (B. dysenteriæ, Shiga)	1	,,
Escherichia coli (B. coli communis)	2 to 5	,,
Proteus vulgaris	10	,,
B. fæcalis alkaligenes	10	,,

When stock vaccines are used, the dose may be two or three times as large as shown above. With acute and generalised infections the initial dose of a vaccine, *if used at all*, should be very small indeed, perhaps not more than one million bacteria, but in cases of prophylaxis it may be given a hundred times as large or more.

With regard to the increment of the dose, some definite system should be followed. The following are the ways by which the dosage may be spaced out: either by the doubling system, e.g., 5, 10, 20, 40, 80, 160, etc., millions; or in pairs of the same size, e.g., 5, 5, 10, 10, 20, 20, 40, 40, etc., millions. Of the two, the doubling system is the better, but even then, successive injections should always be guided by the nature of the reaction provoked by the previous injection.

Methods of administration:—The common method of administration is by hypodermic injection. The dose is repeated usually after 3 to 4 days or a week. In acute cases when the smallest dose is used, it may be repeated after 12 to 24 hours, as the positive phase lasts for a very short time.

Indications for omitting or postponing the inoculation:—

1. Intercurrent illness.
2. A long journey or any other exhausting influence.
3. A menstrual period.

Effects of vaccine injection:—

Three kinds of reactions are possible after a vaccine injection:—

(i) *Local:*—At the site of injection, an inflammatory reaction occurs.

(ii) *General:* Constitutional disturbances, such as pyrexia, headache and a general malaise often result.

(iii) *Focal:* At the site of the lesion, an exacerbation of the inflammatory reaction takes place. Such a reaction is probably specific.

Phases of vaccine treatment

(a) *NEGATIVE PHASE:*—This follows immediately after the injection and lasts for a few hours to a week or 10 days. At this period the person becomes more susceptible to the disease owing to the lowering of immunity, as judged by Wright's opsonic index which is found to be distinctly lowered, the bacterial antigen having used up some of the pre-existing immune bodies. Hence, the second injection should not be given during this period.

(b) *POSITIVE PHASE:*—This follows after the negative phase is over and results in the production of immunity and the consequent increase in the opsonic index.

Another point to be noted is that in addition to the specific response, there appears also to be a non-specific response to the injection of almost any kind of vaccine. In some cases, the good results that are obtained by the use of a vaccine may be attributed to this non-specific response. The best example is the use of non-specific vaccines in the treatment of chronic joint troubles of gonococcal origin. To obtain the best specific response, it is always advisable to select the smooth colonies for the preparation of vaccines.

Purposes for which vaccines are used:—

1. Prophylactic.
2. Curative.
3. Provocative.

Prophylactic:—Vaccines are most commonly employed for the prevention of certain infective diseases in order to increase the resistance of the body against infection. Prophylactic inoculation is a very useful method in controlling the spread of epidemic infectious diseases. The diseases in which prophylactic vaccination has produced marked success are typhoid and paratyphoid fevers, cholera, plague, whooping cough (pertussis), etc. The dosage and the methods of administration are described under respective bacteria.

Curative:—Vaccines, as a method of treatment, are used in certain diseases, particularly in subacute and chronic cases. In acute cases, it is doubtful whether vaccine administration, as a method of treatment, is at all justifiable. In fact, it may be harmful, because the patient's system is highly sensitised to the invading organisms and it would be unwise to tax the system which is already over-stimulated. If vaccines are to be used in acute cases, they should be given in the smallest doses possible, so as to avoid the negative phase altogether and to repeat the injection within a short interval.

Provocative:—By this method, a response (focal reaction) is provoked at the site of the lesion and in this way, any latent focus that might have been present is stirred up. The discharge resulting from such a focal reaction often reveals the presence of the organism which is not detected otherwise and hence, such provocative injections can be utilised as a method of diagnosis. It is particularly employed for the diagnosis of latent gonococcal infection.

Guide to vaccine therapy:—

1. Always make an accurate diagnosis of the disease and the infecting organism.

2. Isolation of the organism in pure cultures is an essential step in the preparations of vaccines

3. Always give preference to *autogenous* vaccines, if they are available.

4. Avoid vaccines which are more than 6 months old or have been subjected to a high temperature or exposed to a strong light.

5. Always begin with small doses. Overdose and much reaction are always bad.

6. Avoid too rapid repetition of injections, as it only exhausts the patient's immunity mechanism.

7. Do not be in an undue hurry when you want to use vaccine therapy, as mistakes are liable to creep in.

8. Do not stop the usual routine treatment, because vaccines cannot replace it. General treatment always improves the patient's system, on which the development of immunity depends to a great extent.

9. If in doubt, whether vaccine therapy is to be done or not, always postpone it.

10. *Always control your vaccine therapy; do not let it control you.*

Varieties of vaccines:—Vaccines may be *specific* or *non-specific.* They are called specific when the organisms used are identical with those causing the disease, as opposed to non-specific vaccines which are employed to produce a protein shock and are not used against any particular infection. The specific vaccines may again be either autogenous or stock, and both may be either simple, when they consist of a single strain, or mixed, when they consist of many strains. Non-specific vaccines are always stock vaccines.

Autogenous vaccines:—These are prepared from the bacterial strain isolated from the lesion in the patient. In order to produce a specific response by vaccine injection, it is always advisable to use an autogenous vaccine.

Stock vaccines: These are prepared from a cultivation of the same species of bacterium isolated and stored in the laboratory, i.e., the vaccine is prepared from the same organism but obtained from a different patient. Stock vaccines are used under the following circumstances:—

(i) For prophylactic inoculation.

(ii) In case of failure to isolate the organism, e.g., in gonorrhœal arthritis.

(iii) Pending the preparation of an autogenous vaccine.

(iv) For the production of " protein shock " (Non-specific therapy).

Mixed vaccines:—They consist of more than one variety of organisms and are used in cases where more than one type of bacteria are believed to be the causal agents, e.g., in respiratory catarrh and in certain suppurative conditions.

Special kinds of vaccines

(a) **Sensitised vaccines** ("Sero-vaccines"):—The bacterial emulsion is treated with its corresponding antiserum and incubated at 37°C. for 2 hours. After this time, the bacteria are separated from the serum by centrifugalisation and are again emulsified in saline in suitable doses and injected. In this way the toxicity is reduced very considerably and a fairly big dose may be given without any untoward effects. This is very suitable for acute and generalised infections, especially by streptococci.

(b) **Detoxicated vaccines:**—These [illegible] so called 'residual vaccines.' They contain the nitrogenous substanc[illegible] the bacteria freed from the endotoxin. The endotoxins are liberat[illegible] disintegration of the bacterial bodies by chemicals, such as alkalis, and the bacterial protein obtained by their subsequent precipitation by acids. Enormous doses may be given without any toxic effects.

(c) **Phylacogens:**—These are preparations of vaccines in which the bacterial bodies are made up in such a way that they are easily assimilated by the tissues. When an ordinary bacterial vaccine is injected, it always takes some time before the bacteria are broken up into products suitable for assimilation. By using phylacogens, we save this time and thereby expedite the action of vaccines. The solution of bacteria is brought about by various agents, physical and chemical. Usually these are made up of various organisms mixed together. Hence they are examples of mixed vaccines.

(d) **Immunogens:**—These are bacterial antigens prepared by extracting the bacteria in normal saline and represent the washings of the outer surface of the bacterial bodies. Immunogens are free from bacteria and their endotoxins, and on this account, do not produce any severe reaction. Hence, they may be administered with greater frequency than is possible with ordinary vaccines for similar purposes. Commonly used immunogens are for Neisseria gonorrhœæ, Diplococcus pneumoniæ, Streptococcus and Hæmophilus pertussis.

CHAPTER XIII

TOXINS AND ANTITOXINS

Historical:—That some bacteria produce an extracellular filterable toxin (*exotoxin*), was first observed by Roux and Yersin (1888) in the case of Corynebacterium diphtheriæ and by Brieger and Kitasato (1891) in the case of Clostridium tetani. In the year 1886, Salmon and Theobald Smith in Washington, working with hog cholera, demonstrated that such diffusible toxins, when inoculated into animals, would produce immunity. A notable advance, however, was made in 1890 by von Behring with his colleagues, Kitasato and Wernicke, who recognised that when diffusible toxins (exotoxins) of C. diphtheriæ and Cl. tetani were injected into animals, in sublethal doses, certain protective substances were developed in the bodies of the inoculated animals which survived the effects of the injections of lethal doses of the toxins. The blood serum of such immunised animals had the power to neutralise the specific toxins and could confer passive immunity to animals suffering from clinical diptheria and tetanus. This was due to the development of certain protective substances in the serum and to the latter the name *antitoxin* was given. In 1891, Pfeiffer first demonstrated with Vibrio comma (V. choleræ) that the toxins did not diffuse out into the culture medium, but remained confined inside the bacterial bodies and were liberated only when the bacteria had undergone disintegration. To such a toxin the name *endotoxin* was applied.

EXOTOXINS AND ENDOTOXINS

Exotoxins (*Exo* means outside):—These are supposed to be secreted or excreted by the bacteria and thrown out into the surrounding medium, the vitality of the bacteria not being injured in the process. Hence, the production of this type of toxin is compatible with the normal life of the bacteria and it is more or less a physiological process. *Corynebacterium diphtheriæ, Clostridium group of organisms, Eberthella dysenteriæ Shiga and Streptococcus scarlatinæ* are commonly known to produce exotoxins.

Endotoxins (*Endo* means within):—These signify internal toxins of the bacteria and are only liberated when the organisms undergo disintegration. Formation of endotoxin has thus been very aptly described by Besredka as a post mortem phenomenon. *Vibrio comma (V. choleræ), Eberthella typhi, Pasteurella pestis, Mycobacterium tuberculosis, Pfeifferella mallei* and a host of other pathogenic organisms are known to produce endotoxins.

EXOTOXINS

Stages in the Preparation:—(i) [illegible] of the bacteria in a suitable *fluid medium* under appropriate [illegible].

(ii) Filtration through a Berkefeld or Pasteur-Chamberland filter.

(iii) Standardisation (*Vide infra*).

Properties:—(i) These toxins are probably nitrogenous substances.

(ii) They are soluble and cannot be crystallised.

(iii) They are dialysable, i.e., can pass through a semi-permeable membrane.

(iv) They are very unstable bodies, and lose their toxicity very rapidly when heated to 70°C. and their potency destroyed on keeping and also when exposed to sunlight and air.

(v) They are precipitated out by many salts, particularly ammonium sulphate. Such precipitated toxins, when dried (as in the cases of Cl. welchii and Cl. tetani), are much more stable than when they are in the liquid state.

(vi) Exotoxins, when exposed to the action of formaldehyde (0·3 per cent.), are modified into a form, designated as *toxoid* by Ehrlich and *anatoxin* by Ramon.

Ehrlich's view on the nature of exotoxins:—As mentioned before in his side-chain theory, Ehrlich considers that a toxin molecule consists of two parts, a haptophore and a toxophore, group. The former combines with the receptor of the cell and is thus anchored to the cell protoplasm, so that the latter can now exert its poisonous action on the cell (Fig. 85). Toxophore group is thermolabile whereas haptophore group is thermostable. It is the haptophore group which is responsible for the elaboration of the specific antitoxin.

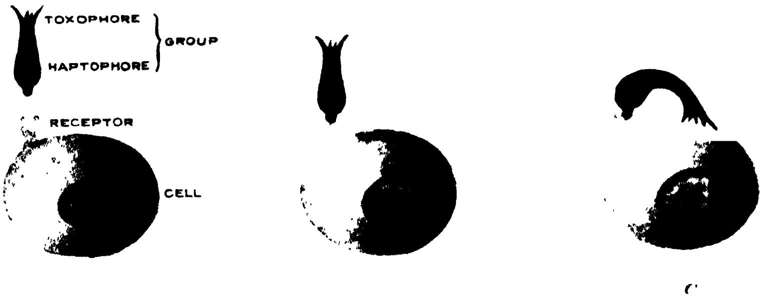


Fig. 85.

Ehrlich's conception about the nature of toxin molecule and its mode of action.

a, a cell with its receptor and a free toxin molecule; b toxin molecule has united with the cell receptor; c, toxophore group is injuring the cell as shown by clearing of the area.

Modified toxin (toxoid or anatoxin):—When a toxin molecule is heated to 65° to 70°C., or exposed to the action of 0·3 per cent. formaldehyde, or preserved for a considerable period, its toxophore group, being unstable, is destroyed, leaving the haptophore group intact. The toxin molecule is thus modified into a form which no longer produces symptoms when injected into an animal. Such an altered toxin, however, having retained its haptophore group, is thus still able to act as an antigen and capable of neutralising antitoxin. This modified toxin is called the *toxoid* (Ehrlich) or *anatoxin* (Ramon's formolised toxin). The properties of a toxoid may be summed up as follows:

(1) It contains only the haptophore group; hence it is stable, has the power to combine with its corresponding antitoxin and to be neutralised.

(2) It is antigenic, i.e., when injected into an animal, it can give rise to the formation of its specific antitoxin.

(3) It does not contain any toxophore group and is therefore non-toxic when injected into animals.

Uses of exotoxins:—(i) For the production of antitoxic sera.

(ii) As a method of active immunisation against scarlet fever and diphtheria (Vide under the respective organisms).

(iii) For diagnostic purposes, e.g., Schick test in diphtheria and Dick test in scarlet fever (Vide under C. diphtheriæ and streptococcus).

ENDOTOXINS

Properties:—Vide page 39.

Uses:—(i). For the production of antibacterial sera. (Such sera do not contain any antitoxin).

(ii) For therapeutic purposes, e.g., tuberculin treatment in cases of glandular, bony and cutaneous tuberculosis (Vide under M. tuberculosis).

(iii) For diagnostic purposes, e.g., tuberculin tests in tuberculosis (von Pirquet's test and others) and mallein test in glanders (Vide under M. tuberculosis and Pf. mallei).

ANTITOXINS

These are specific antibodies and belong to Ehrlich's receptors of the first order. They are closely related to the globulin fraction of the serum. The antitoxin may be precipitated out of the serum by partial saturation with ammonium sulphate. Thus, by "salting out" globulin constituents of the serum, the antitoxin can be precipitated and recovered in a concentrated form. The potency of the antitoxin is diminished to some extent with ageing of the serum. When saturated with common salt, the serum remains sufficiently stable. The addition of 50 per cent. glycerol helps to stabilise antitoxin. Phenol has a much more destructive action on the antitoxin than on toxin and it should not be used as a preservative for antitoxic serum. Antitoxins have a molecule of greater size than the toxins, and hence, the former are much more slowly absorbed when injected subcutaneously than is the case with toxins.

Fate of the antitoxin introduced into the body artificially:—The time required for attaining the maximum concentration in the blood depends upon the *route* by which the antitoxin is administered. Thus, when introduced by the subcutaneous route, it takes about 3 to 4 days, by the intramuscular route, 12 to 24 hours and by the intravenous route, immediately.

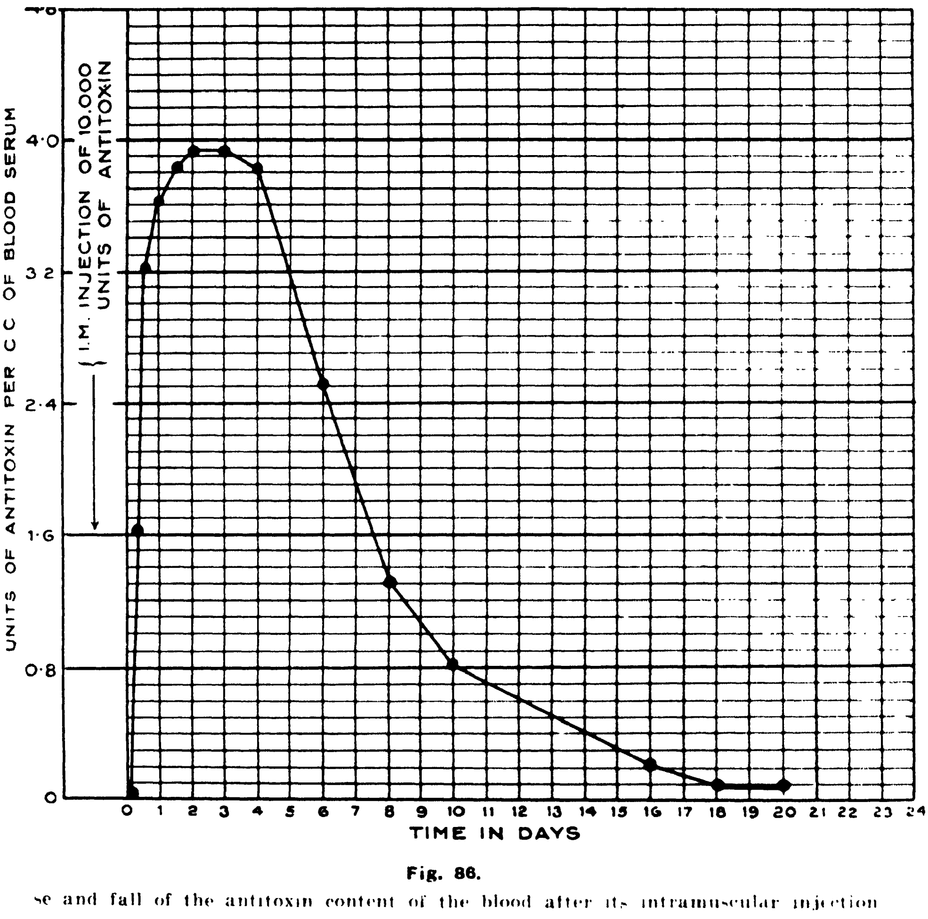


Fig. 86.

se and fall of the antitoxin content of the blood after its intramuscular injection

As soon as the maximum concentration of the antitoxin is reached in the circulating blood and tissue fluids, and the amount necessary for neutralisation of toxin is utilised, the rest is rapidly eliminated from the system and in about three weeks' time, it completely disappears. If the animal had received an injection of horse serum previously, the antitoxin is eliminated much more rapidly. The rise and fall of antitoxin in the blood, after artificial inoculation, can be represented graphically as shown in Fig. 86.

Elimination of the antitoxin from the body is effected through the various secretions, where it is found in much less concentration than in the blood. Milk contains some antitoxin and therefore a certain degree of immunity may be conferred to sucklings, by feeding them with such milk. As mentioned before, diphtheritic antitoxin may thus be transferred to the new born babies, partly by the mother's milk which may be sufficient to give them immunity against diphtheria during their first year of lives. This amount, however, gradually diminishes and, unless otherwise developed, these children again become susceptible to diphtheria. Hence the incidence of this infection is greatest during 2 to 5 years of age.

Sources of antitoxin:—Like all antibodies, the main source of antitoxin formation is the reticulo-endothelial system. The genesis of antitoxin has already been discussed on page 137-138.

Stages in the preparation of antitoxin:—

1. Preparation of the standard toxin.

2. Selection of an animal. Usually, in most cases, horses are selected for the manufacture of antitoxic sera but goats are used in the case of Clostridium welchii. Rabbits may be used for smaller quantities.

3. Immunisation of the animal by repeated injections of suitable doses of the toxin.

4. Bleeding and collection of the antitoxic serum.

5. Standardisation (*Vide infra*).

Uses of antitoxin:—For passive immunisation.

(i) It is most commonly employed for *therapeutic purposes*. In order that the treatment may be effective, the antitoxin should be administered at the earliest possible moment, in sufficient amount and by the quickest route possible. Antitoxin neutralises the toxin that is present in the blood and tissue fluids and thus prevents the injurious action of the toxin, but it cannot undo the damage that has already been produced.

(ii) *Prophylactic use* is very limited and is particularly used in tetanus and gas gangrene. When administered for this purpose, the antitoxin counteracts the effects of the infecting organisms, but it cannot prevent the infection. The value of antitoxin prophylaxis is therefore to neutralise the poisonous effects of the supposed infection, thus allowing the invading organisms to fall a natural prey to the defensive forces of the body (cells and fluids) and to be eliminated from the system in this way. As the antitoxin disappears from the blood after some time, it is necessary that prophylactic administration of antitoxin should be repeated at intervals of 5 to 6 days.

UNITS OF MEASUREMENT OF TOXINS AND ANTITOXINS

In the early days of immunology the strength of a toxin was measured by the *minimal lethal dose (M.L.D.)* of Ehrlich. The M.L.D. of a toxin is defined as the smallest amount of toxin which, when injected subcutaneously into a guinea-pig of 250 gms. weight, will kill the animal within 4 days. The *unit of antitoxin* as defined by Ehrlich is the amount which will completely neutralise the effect of 100 M.L.D. of a particular toxin, i.e., prevent the death of a guinea-pig of standard weight within the specified time.

It was soon realised by Ehrlich, that the value of M.L.D. of a particular toxin diminishes on keeping, owing to the formation of toxoid. The toxoid, which is always present in a preparation of filtered broth culture in association with the toxin, also plays an important part in combining with the antitoxin and hence the above measurement was replaced in many laboratories by Lo or L+ test. This is measured by the combining power of the total amount of specific antigen (toxin and toxoid) with 1 unit of antitoxin. The combining power of toxin and antitoxin is tested by injecting the mixture subcutaneously into a series of guinea-pigs of 250 gms. weight and two convenient end points are found out—(i) the death of the guinea-pig within 4 days, which gives the L + dose (Limes Tod or limit of death) and (ii) absence of local œdema, which gives the Lo dose (Limes Nul or limit of no reaction).

The *L + dose* of a toxin is defined as the lowest amount of toxin which, when added to 1 unit of antitoxin and injected subcutaneously into a standard guinea-pig, will kill the animal within 4 days. Such a quantity is expressed by Ehrlich as "Limes Death" (Lime boundary or threshold) or briefly L+.

Similarly, *Lo dose* of a toxin is defined as the highest amount of toxin which, when added to 1 unit of antitoxin and injected subcutaneously into the guinea-pig, will cause no observable reaction either local or general. Such an amount is expressed by Ehrlich as "Limes Zero" or briefly Lo.

Thus it will be seen that L+ dose of a toxin plus 1 unit of the corresponding antitoxin contains some amount of toxin uncombined, which kills the guinea-pig and this may be equivalent to 1 M.L.D., whereas the mixture of Lo dose of the toxin and 1 unit of its antitoxin is completely neutral; therefore the difference between L+ dose of a toxin and Lo dose must be equal to 1 M.L.D. But in actual practice, it is always found that the amount (L+ minus Lo) is greater than 1 M.L.D. and may be anywhere between 10 and 100 M.L.D. This is known as ***Ehrlich's phenomenon.***

Antitoxin is now-a-days preserved in the three standard laboratories in England, United States of America and Germany, as a dry powder, without containing any antiseptic and keeping it *in vacuo*. When required, a measured quantity of this dried antitoxin is dissolved in 50 per cent. glycerin and distributed to serum manufacturers to check their test toxins. The three standard laboratories are (i) National Institute for Medical Research, London, (ii) The Hygiene Laboratory, U.S.A. Public Health Service, Washington and (iii) The Institut für experimentalle therapie, Frankfurt, Germany.

On account of the instability of toxins, a new antitoxic serum cannot be standardised with it, but is compared with a standard antitoxic serum through the intermediary of its toxin. This is done by first finding out L+ dose of the toxin, for which a new antitoxic serum is obtained, and which is determined by a standard antitoxic serum. After finding out the L+ dose of the toxin, it is necessary to determine the amount of the new antitoxic serum that will prevent death of a standard guinea-pig within 4 days, when injected together with a L+ dose of the toxin. Thus the unit of the new antitoxin is determined as the amount which will prevent the death of a standard guinea-pig within 4 days when injected together with L+ dose of that particular toxin.

As the method of standardising toxins and antitoxins by killing guinea-pigs is an expensive one, for economy, the intracutaneous (intradermal) method of Römer and Sames (1909) was introduced. By this method all the test animals survive and can be used for other purposes. This method led to the use of two other standard units, viz. (i) M.R.D. (minimal reacting dose) and (ii) Lr (limit of reaction). The *minimal reacting dose (M.R.D.)* of a toxin is defined as the lowest amount which, when injected intracutaneously into a guinea-pig, causes a minimal skin reaction in the form of a small red flush, 5 m.m. in diameter, visible 36 hours after the injection. The *Lr (limit of reaction) dose* of a toxin is defined as the lowest amount of toxin which, when mixed with 1 unit of antitoxin, forms a mixture of which 0·2 c.c., injected intracutaneously into guinea-pigs, causes a minimal skin reaction. The antitoxins can now be standardised in terms of this Lr dose of the toxin, on the same line as in the previous method.

Fraser and Wingham (1924) recommend the use of rabbits in place of guinea-pigs for the intracutaneous test of diphtheria toxin. The skin of this animal is very sensitive to the action of diphtheria toxin and the same animal may be utilised for a large number of samples and thus giving an advantage of economy of time and animal.

Recently, there is yet another method of estimating the strength of toxin and antitoxin without the use of experimental animals. This is *in-vitro method of Ramon's flocculation test.* This method depends on the fact that when, to a series of test tubes, a constant amount of toxin and varying amounts of undiluted antitoxic serum are added, the combination of toxin with antitoxin is accompanied by a flocculent precipitate. The flocculation first appears, and occurs rapidly in the tube in which the mixture is exactly balanced (neutral) and spreads to adjacent tubes in both directions. The tube in which there is an excess of toxin or antitoxin shows no flocculation. This test determines the total combining capacity of the filtrate (toxin and toxoid) for antitoxin and not the amount of toxin only. Owing to the ease and rapidity with which the test is carried out, it has an additional advantage over animal experimentation. The unit first suggested by Ramon was "antigenic unit" but it has been changed to Lf unit (Limit of flocculation) in conformation with other units of toxins

The *Lf dose* of a toxin is defined [illegible] amount of toxin which flocculates with a maximum rapidity wh[illegible] with one unit of antitoxin.

The following table summarises the various methods by which the units of toxins are measured:—

Nature.	Method.		Substances required.	Reactions.	Units.
Measuring specific toxicity i.e. Toxin content alone.	In vivo.	Subcutaneous.	Least quantity of toxin.	Kills a guinea-pig of 250 gms. wt. within 4 days.	M. L. D. (Minimal lethal dose).
		Intracutaneous.	Least quantity of toxin.	Causes minimal skin reaction in the form of a small red flush of 5 m.m. diameter, visible after 36 hours.	M. R. D. (Minimal reacting dose)
Measuring combining power with 1 unit of antitoxin i.e. Toxin + Toxoid content.	In vivo.	Subcutaneous.	Least amount of toxin + 1 unit of antitoxin.	Kills a guinea-pig of 250 gms. wt. within 4 days.	L+ (Limit of death)
		Subcutaneous.	Largest amount of toxin + 1 unit of antitoxin.	Causes no local oedema	Lo (limit of no reaction)
		Intracutaneous.	Least amount of toxin + 1 unit of antitoxin; 0.2 c.c. of this mixture is injected.	Causes minimal skin reaction in the form of a small red flush of 5 m.m. diameter, visible after 36 hours.	Lr (Limit of reaction)
	In vitro.		That amount of toxin + 1 unit of an antitoxin.	Flocculates most rapidly.	Lf (Limit of flocculation).

Note: There is no direct method of measuring the toxoid content alone.

ACTION OF TOXINS

When toxins are injected into the circulation, they reach every cell in the body, including the susceptible ones, and enter into a sort of loose combination which may be easily dissociated. It is really a sort of adsorption by the cells. In this stage, actual damage does not take place. Later, the toxin enters into a firm combination with the susceptible cells and after once this has taken place, it cannot be dissociated. It is only after such a firm union has occurred, that the toxin is enabled to exert its specific toxic effect. Unless the toxin combines with the cells, neither can it produce its poisonous effects nor can it give rise to the formation of antitoxins. This is shown by the following experiment:—

Rat is non-susceptible to diphtheria toxin and when the latter is injected into the circulation of the animal, it remains free and uncombined in the blood stream for a long time, so that its blood is toxic to a susceptible animal, such as a guinea-pig. This is due to the fact that owing to the absence of a suitable receptor, the toxin is not absorbed by the tissues and hence not removed from the circulation. In this animal no antitoxin is produced. Similar experiments may also be performed with tetanus toxin in case of a turtle. In some cases, however, though a firm union occurs, no damage is inflicted to the cells, although production of antitoxin takes place. This can be demonstrated in the alligator which is non-susceptible to tetanus toxin, though the latter combines with its cells. As the production of antitoxin occurs only in individuals whose cells have the specific power of binding the toxin, the alligator is capable of producing tetanus antitoxin.

Practical application of the above knowledge

(1) As there is a long latent period which must elapse before the toxin can produce its injurious action, antitoxins should be introduced in the treatment of an infection as early as possible.

(2) The union between the toxin and the tissues is at first a loose one and a large quantity of antitoxin can dissociate such a union during its initial stage. Hence the necessity of the use of antitoxin in large excess. Once the union is complete and the cells are damaged, no amount of antitoxin can undo the mischief that has occurred.

ON THE NATURE OF UNION BETWEEN TOXIN AND ANTITOXIN

Toxin and antitoxin unite outside the body (*in vitro*) to form a distinct compound, but this union, as will be seen later, is not immediate. During such a process the toxin is not destroyed by the antitoxin, as there is evidence to show that the toxin-antitoxin complex, when formed, may afterwards be dissociated and the toxin again liberated.

Proof that toxin-antitoxin complex is dissociable:—

1. Calmette's experiment with snake venom and its corresponding antitoxin (antivenin):— Snake venom is thermostable, not being destroyed at 80°C., whereas its antitoxin is rendered inert at much lower temperatures (68°C.). He found that a neutral mixture of venom and antivenin becomes toxic again, if heated to 68°C. for 10 minutes. The antivenin has been destroyed by heating, but not the venom, although it had combined with the former.

2. Use of toxin-antitoxin mixture for immunising children against ia, resulted in some deaths. The [illegible]gation into this untoward

result of inoculation subsequently proved that the toxin was dissociated from its combination with the antitoxin, while the toxin-antitoxin mixture was kept in the cold chamber.

It has been found that the interaction between toxin and antitoxin takes some time and if sufficient time is allowed for this, the union may be a firm one. This is proved by the filtration experiment of Martin and Cherry and supported by Brodie. They made use of porcelain filters, the pores of which were blocked with gelatin. Such a filter will allow the smaller toxin molecules to pass through, but not the antitoxin molecules which are much larger than the former. If a mixture of toxin and antitoxin is made and is allowed to pass through such a gelatin-saturated filter candle at various periods after mixing, it is found that the amount of toxin that can pass through the candle is less, the longer the time elapses between the mixing and filtration. If the mixture is filtered after it is allowed to act for 2 hours, the filtrate will be altogether non-toxic, showing that firm combination of toxin and antitoxin has taken place. Similarly, in Calmette's experiment with snake venom stated above, if the mixture is allowed to act for some time, say for more than 30 minutes, it will be far more difficult to liberate the venom from the combination and in that case heating of the mixture will not cause any toxicity.

The above experiments show that union of toxin and antitoxin takes place slowly and is at first a loose one, i.e., dissociable, but with lapse of time, the union becomes firmer and firmer.

Danysz phenomenon:—It is found that a given amount of antitoxin neutralises less toxin when the latter is added in several fractions at intervals than when all the toxin is added at once. This was first noted with ricin and antiricin by Danysz (1902) and later confirmed by von Dungern (1904), using diphtheria toxin and antitoxin.

Nature of union between the toxin and the antitoxin:—It is admitted that the toxin and the antitoxin combine to form a distinct compound but there is still much uncertainty as to the nature of this union. Regarding this subject, there are two main theories, chemical and physical. The former is advocated by Ehrlich and modified by Arrhenius and Madsen, and the latter by Bordet and further extended by von Krogh.

1. *CHEMICAL THEORY:*—Ehrlich believes that there is a firm chemical union between toxin and antitoxin, just as a strong acid combines with a strong base, conforming with the law of multiple proportions. Arrhenius and Madsen suggested that the interaction between toxin and antitoxin is like that between weak acids and weak bases, which is governed by the law of "mass action" and is apt to exhibit a reversible action. From this it will follow that the union is unstable and easily dissociated. In a neutral mixture of toxin and antitoxin, there is always present free toxin and antitoxin as well as toxin-antitoxin complex. This theory thus explains the Ehrlich's phenomenon but fails to explain the Danysz phenomenon.

2. *PHYSICAL THEORY:*—In order to explain the various phenomena of toxin-antitoxin reactions, Bordet introduced his physical theory of adsorption and suggested that a molecule of toxin is adsorbed by its specific antitoxin, exactly in the same way as a dye is adsorbed on to a filter paper. In a mixture of toxin and antitoxin, the latter is equally distributed over the molecules of the former, forming a uniform mixture of partially saturated toxin or partially saturated antitoxin, according to the proportion of the two substances. Once the antitoxin has combined with the toxin, it is very difficult for the latter to combine again with further antitoxin and in this way Danysz phenomenon can be explained. von Krogh further extended the adsorption theory of Bordet. He regarded that toxin-antitoxin reaction takes place in two stages. In the first stage, toxin is fixed to the antitoxin by adsorption. In the second stage, after this union, a secondary chemical combination occurs and a compound is formed, resulting in a flocculent precipitate.

Conclusion:—It is to be seen that Bordet's adsorption theory, with the extension of secondary chemical combination of von Krogh, can explain all the phenomena of toxin-antitoxin reaction.

CHAPTER XIV

AGGLUTININS AND AGGLUTINATION REACTION

In 1889, Charrin and Roger noted that when bacteria were allowed to grow in the serum of immunised animals, they formed clumps which were deposited at the bottom of the vessels. It was Grüber and Durham (1896), however, who first made a special study of the phenomenon of specific agglutination. In June, 1896, Widal showed that the blood serum of a patient suffering from typhoid fever developed specific agglutinins during the course of the illness and suggested that agglutination reaction might be of great value for diagnostic purpose in typhoid fever. The reaction has therefore been known as Widal's reaction.

Definition:—Agglutinins are antibodies which are developed in an immune serum and by virtue of which, bacteria are made to aggregate into clumps when suspended in a saline solution. With this clumping, the bacteria, if previously motile, become motionless. Such a phenomenon may be observed with non-motile, as well as dead, bacteria; hence, it is not necessary that living and motile organisms should be used for the reaction. Agglutinins, apart from being developed in the sera of immunised animals, are formed in the blood of patients suffering from certain bacterial diseases, such as enteric fever, Malta fever, etc. The substance which excites the formation of agglutinins in the animal body is called the agglutinogen.

Properties of agglutinins:—

1. They are very stable bodies, withstand drying for many months, even when freely exposed to the air, are little affected by light and putrefaction and not destroyed by heating at 55°C.

2. They act best in a neutral or faintly acid medium and are injured by alkalis.

3. Like most other antibodies, they are closely related to the globulin fraction of the serum.

4. The presence of the complement is not necessary for their activity.

5. Ehrlich regards agglutinins as receptors of the second order, consisting essentially of two chemical side-chains, a haptophore group combining with the antigen and a zymophore or ergophore group which causes the clumping (Fig. 87). The latter is more easily destroyed than the former which is heat-resistant (cf. toxin). A serum containing agglutinin, when heated to 70°C., is unable to cause clumping of its homologous antigen (bacterium) as its zymphore group is injured, but is still capable of uniting

with its specific antigen. To such an inactive agglutinin the term *agglutinoid* is applied (cf. toxin and toxoid). Agglutinoid has a greater affinity for the bacterial receptors than has agglutinin. This will be referred to later in explaining the *"zone phenomenon"* of agglutination reactions.

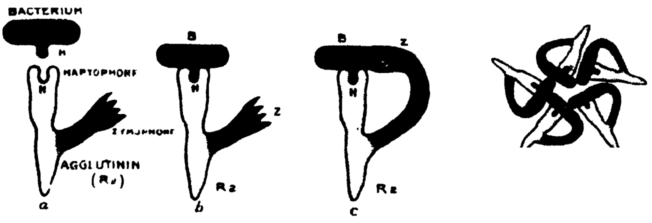


Fig. 87.

Ehrlich's conception about the nature of agglutinin and its mode of action. (A diagrammatic representation).

Nature of the agglutination process:—There are many views as to the nature of the union between agglutinin and agglutinogen. That agglutination process is purely a physical phenomenon was distinctly shown by *Bordet*. He considers that the reaction occurs in two phases. In the first phase, the agglutinin unites with the agglutinogen, i.e., the bacteria adsorb the specific agglutinin and are thus sensitised. While in the second phase, the sensitised bacteria, in the presence of electrolytes, agglomerate ,into clumps and settle down by gravity. This stage can be postponed indefinitely by keeping the mixture of the bacteria and diluted serum, as free as possible, from electrolytes. It is to be noted that as a result of union between the agglutinin and agglutinogen, some physical alteration is produced in the bacteria, permitting them to be agglutinated in the presence of electrolytes.

Effect of agglutination:—The vitality of the bacteria is in no way injured by their specific agglutinins. On the contrary, they are quite capable of multiplying and growing in the agglutinated state. The value of agglutination as a means of resistance to infection, is not yet settled; but possibly some degree of agglutination may facilitate phagocytosis and localisation of the infection.

Varieties of agglutinins:—When bacteria are introduced into the system, they may cause the development of not one, but several different types of agglutinins. The bacterial cells may be regarded as consisting of complex substances, all or some of which act as antigens and form agglutinins. Several units of such complex bacterial cells may also occur in the closely related bacteria of different strains. Such a conception of the composition of bacterial antigen is known as the " *antigenic mosaic.*" This feature is specially noticeable in the case of the dysentery bacillus of Flexner (*vide infra*).

Agglutinins which act on the infecting organisms are known as the *primary, homologous, specific or major agglutinins*. This type of agglutination is observed with relatively high dilutions of the homologous serum, the specific antigen alone exhibiting the clumping. Agglutinins which act on other closely related bacteria are known as *secondary, heterologous, group or minor agglutinins.* This type of agglutination is only observed with lower dilutions of the serum, and it is non-specific in character. In some cases, particularly with the Salmonella organisms, the group agglutination may be so marked that it is impossible to identify the different strains of the group by the use of the agglutinating serum even at full titre (Titre is the highest dilution at which agglutination occurs). The organisms can, however, be differentiated by the use of the *absorption or saturation test of Castellani and by cross agglutination.* This is determined by saturating the agglutinating serum with the culture of the homologous organism, centrifuging, and then testing whether the supernatant fluid will agglutinate the organisms of the allied strain.

RECENT RESEARCHES ON AGGLUTINOGENS AND AGGLUTININS

Within recent years, a number of discoveries has been made regarding the varieties of agglutinogens and agglutinins. It has been recognised that different components of a bacterial cell may behave as separate agglutinogens, giving rise to their specific agglutinins. In the case of motile bacteria, it has been found by Theodore Smith and Reagh (1903) that flagella took an important part in agglutination, in addition to bacterial bodies. Thus, two kinds of aglutinins have been detected; that produced by flagella is called *flagellar or H agglutinin,* and that produced by the bacterial body is called *somatic or O agglutinin.* This is known as qualitative receptor analysis. Again, a specific agglutinin has been found in association with the capsule or the coat surrounding the bacteria, e.g. in Diplococcus pneumoniae. Other kinds of agglutinins are observed in connection with variation of colonies from smooth to rough forms, e.g. in the case of Eberthella typhi (Arkwright).

Flagellated bacteria:—

FLAGELLAR OR H AGGLUTININ AND ITS AGGLUTINOGEN:— Flagellar or H agglutinin forms clumps which appear much more quickly and are large, loose, fluffy and flocculent. These clumps are easily broken on shaking. When they are examined microscopically, they are found to be formed by the cohesion and interlacing of the flagella. The bacilli are found not touching one another, but lying in a tangled mass of agglutinated, motionless flagella (Fig. 88). These agglutinins are heat-resistant, i.e., not destroyed by heating at 60° to 62°C. As will be seen later, these H agglutinins, being heat-resistant, can be separated from the O agglutinins which are heat-labile, by heating. They can also be separated by the absorption test, i.e. saturating the serum with a suspension of non-motile strain which will remove all the somatic or O aggultinins but leave the H agglutinins untouched.

Its corresponding antigen (H or flagellar agglutinogen) is destroyed by heat at 60° to 62°C., or by mixing it with alcohol. Similarly, by growing the bacilli in 0·1 per cent. phenol agar, the flagellar or H antigen is reduced or abolished, leaving only the somatic or O antigen.

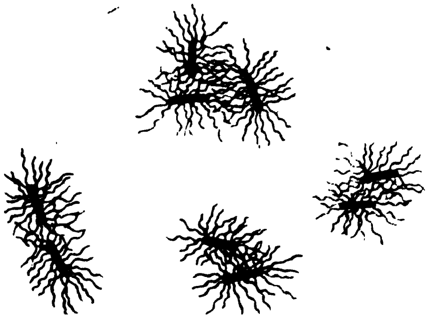


Fig. 88.
Flagellar agglutination under the microscope.
(A diagrammatic representation).

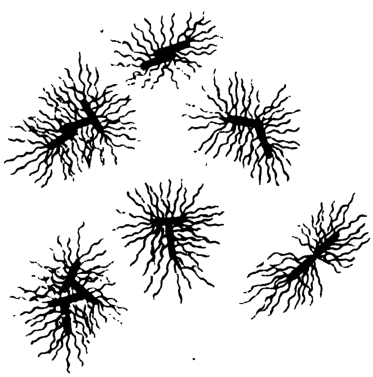


Fig. 89.
Somatic agglutination under the microscope.
(A diagrammatic representation).

SOMATIC OR O AGGLUTININ AND ITS AGGLUTINOGEN:—In the process of agglutination by somatic agglutinins, smaller and denser clumps are formed very slowly, and they appear as small granules which may be of the size of grains of sand, or too small to be detected by the naked eye. The clumps cohere much more firmly and are not nearly so readily affected by shaking as the flagellar ones. When these clumps are examined microscopically, the bacterial bodies are found to be in close contact with one another, quite unlike the picture found in flagellar agglutination (Fig. 89). Such clumps may move about by the action of the free unagglutinated flagella. Somatic or O agglutinins are thermolabile, i.e. less heat resistant than the H aglutinins. In this connection, it should be noted that O agglutinins of group organisms are all similar and identical, whereas the H agglutinins are specifically different.

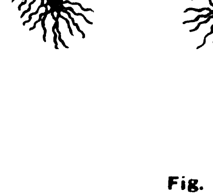


Fig. 90.
Diagram showing the loss of flagellar antigen H when the normal motile form is transformed into a non-motile one.
M.S. Motile smooth form; N.S.- Non-motile smooth form.

Its corresponding antigen (O agglutinogen), unlike H antigen, is neither destroyed by heat nor by alcohol. Hence, for carrying out the somatic agglutination, its specific antigen is prepared by treating the bacterial emulsion, either with alcohol or heat, thereby destroying the flagellar or H antigen, while leaving the somatic or O antigen intact. Somatic antigen can also be obtained by favouring the growth of non-motile forms, as is done by growing the organisms in 0·1 per cent. phenol agar. The O forms so produced are eventually non-motile and devoid of flagella (Fig. 90).

NOTE:—The words H and O represent the German names *Hauch* and *Ohne Hauch*, meaning spreading and non-spreading growth of Proteus vulgaris in which organism these antige[illegible] originally described by Weil and Felix.

VALUE OF SOMATIC AND FLAGELLAR AGGLUTININS

1. *In diagnosis:—*

(i) Flagellar agglutinin is not present in normal serum whereas somatic agglutinin may be present in it in a very low titre.

(ii) In the process of infection with motile organisms, O agglutinins appear earlier than H agglutinins (Felix). As already referred to, the O agglutination is not a specific but a group reaction, being common to the allied organisms. Hence, for the differentiation of the species in the group, one must wait for the appearance of the H agglutinins.

(iii) In a well developed antiserum, the flagellar agglutination may occur in a much higher dilution (1 : 20,000) than somatic agglutination (1 : 200).

(iv) The effect of the administration of a vaccine results in the production of the H or flagellar agglutinins which cause floccular agglutination of the bacillary emulsion with a very dilute serum, but the O or somatic agglutinin, which is responsible for granular agglutination, is either absent or present only in a very low titre. In a vaccinated person, although these antibodies may disappear from the serum in course of time, a very slight stimulus, be it specific or non-specific, causes their reappearance owing to the reactive condition of the cells which produced them. The reappearance of antibodies in response to such non-specific stimulus is called the *anamnestic reaction*, and this occurs chiefly with flagellar or H type of agglutinin; somatic or O type of agglutinin is not capable of resuscitation by the non-specific stimulus. Thus, somatic agglutinin, though less specific, is of very great value in the diagnosis of an infection, particularly in the inoculated persons. By relying on the O type of agglutination, errors in diagnosis are not likely to occur.

2. *As an index of immunity:* Until lately, the development of a high degree of agglutinin in the serum of a patient infected with Eberthella typhi was considered to have no relation with the question of immunity. This was because all the attention was paid only to the H or flagellar agglutinins. Now-a-days, the inception of the somatic or O agglutinins in this field has altered the previous idea. This, as already pointed out, is the result of a specific reaction associated with the development of immunity. Thus, it will be seen that the presence of somatic or O agglutinins in the serum of a patient suffering from E. typhi infection always signifies a good prognosis. In this connection, it should be noted that according to Felix, the most severe and fatal cases of typhoid fever do not produce any O agglutinins.

The properties of two types of agglutinins and agglutinogens may be summed up in the following table:—

Flagellar or H agglutinin.	*Somatic or O agglutinin.*
1. Clumps appear quickly; large, loose, fluffy and flocculent. They are easily broken.	1. Clumps are formed slowly; small and dense. They are not easily broken.
2. Heat-resistant.	2. Heat-labile.
3. Specific.	3. Not specific but a group phenomenon.

	Flagellar or H agglutinin.		*Somatic or O agglutinin.*
4.	Not present in the normal serum.	4.	Present in the normal serum in low dilution.
5.	Appears late in an infection.	5.	Appears earlier in an infection.
6.	Develops during prophylactic vaccination.	6.	Either absent or present only in very low dilutions, during prophylactic vaccination.
7.	Capable of responding to non-specific stimulus.		Not capable of responding to non-specific stimulus.
8.	Not an index of immunity.	8.	An index of immunity.

	H agglutinogen.		*O agglutinogen.*
1.	Related to flagella.	1.	Related to bacterial bodies.
2.	Destroyed by heat (heat-labile).	2.	Not destroyed by heat (heat-resistant).
3.	Destroyed by alcohol.	3.	Not destroyed by alcohol.

MULTIPLICITY OF SOMATIC AND FLAGELLAR ANTIGENS:—Recent works have tended to show that certain bacteria may contain more than one flagellar and somatic antigens. That the somatic antigen is often complex and consists of several units, forming what has been called the *antigenic mosaic*, is best illustrated in the case of dysentery bacillus of Flexner. Since this bacillus is non-motile, only O type of agglutination is to be observed and this is particularly so with the Shiga's strain which contains only one agglutinogen and for which one agglutinin is found in the corresponding serum. But in the case of Flexner's strain, Andrews and Inman (1919) were able to demonstrate the presence of five or more somatic antigens, which enabled them to classify this species into several strains, such as V, W, X, Y and Z.

In the case of the Salmonella group, the flagellar or H antigen is diphasic, one phase being specific for a particular type, the other or group phase being shared by a number of related types.

Capsulated bacteria:—The capsulated bacteria, e.g., Diplococcus pneumoniæ, however, give rise to two types of agglutinins, one associated with the capsular substance (a polysaccharide) which gives the type-specificity and the other with the bacterial endoplasm (a nucleoprotein) which determines the species characters. The serum of an animal immunised with capsulated D. pneumoniæ, agglutinates only cocci of the same type, whereas the serum of animals inoculated with the cocci devoid of capsules agglutinates all D. pneumoniæ, whatever be their type. This subject is dealt with in greater detail under D. pneumoniæ.

Colony Variation in Relation to Agglutinins:—It is now known that two types of colonies grow in connection with certain motile bacteria. These are the smooth or S type of colony, representing the ordinary form and the other, the rough or R type. Such transformation from S to R types is attended with an alteration in the structure of the somatic antigen which is now designated as Ø antigen (Fig. 91). When this alteration takes place in the antigenic structure, its specific character disappears, and, instead of being confined to one genus, the O agglutinogens become cosmopolitan, being distributed widely in different genera of bacteria. For example, in the case of Eberthella typhi, when the normal smooth type of bacilli changes into the rough type, the corresponding somatic O antigen also changes into Ø variety, which is no longer confined to the Eberthella group but is also found in other bacterial species. Thus, along with the colony variations, the specificity of the agglutinogen (antigen) is also lost. Another point is to be noted that an emulsion of rough colonies tends to autoagglutinate in physiological salt solution.

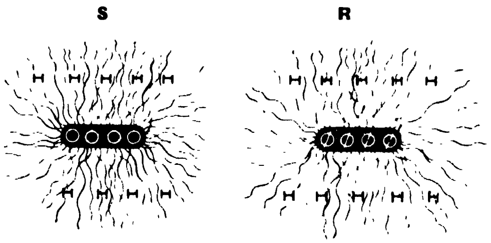


Fig. 91.

Diagram showing the antigenic structure of *Eberthella typhi* in al and rough forms; note the alteration of th antigen. There is however no chan flagellar antigen. S represents the sm and R the rough form.

USES OF AGGLUTINATION REACTION

1. ***For diagnostic purpose,*** specially in the following diseases, such as enteric group of fevers, undulant fever (Malta fever), bacillary dysentery and cholera. In these cases, the agglutinating power of the patient's serum is determined upon the culture of a known organism supposed to be the infecting agent. The reaction is associated with the name of Widal and is called *Widal's reaction.* It was first used by him in the diagnosis of typhoid fever.

2. ***To identify an organism*** that has been isolated from an infected material. This is particularly applied in cases of E. typhi, Salmonella group of organisms, E. dysenteriæ, V. comma, etc. This reaction is known as *Bordet-Durham reaction.* For this purpose the suspected organism, which has been isolated, is tested for its agglutinability against a known serum of high titre, i.e., high agglutinating value (at least 1 : 1,000). Such high titre sera are prepared in the laboratory by inoculating rabbits with the specific organisms.

3. ***To establish a serological classification*** of bacterial strains, e.g., of Diplococcus pneumoniæ, Neisseria meningitidis and others.

DEMONSTRATION OF AGGLUTINATION REACTION

Agglutination of bacteria by specific antiserum may be demonstrated by two chief methods, microscopic and macroscopic (by the naked eye). Whichever method is used, the three reagents which are always necessary for the reaction are serum, bacillary emulsion and a suitable electrolyte (normal saline).

Microscopic method:—Three dilutions of the serum, 1 : 10, 1 : 20 and 1 : 50, are prepared in test tubes or watch glasses by the drop method. This may be conveniently done by taking 1 drop of the serum and mixing it with 9 drops of normal saline, making a dilution of 1 in 10. One drop of this diluted serum, when mixed with one and four drops of normal saline, will give a dilution of 1 in 20 and 1 in 50 respectively. When a drop of each of these diluted sera is mixed with a drop of the bacillary emulsion, the final dilutions of 1 in 20, 1 in 40 and 1 in 100 are obtained. It is preferable to have a young live broth culture for this test, as clumping of the bacilli is more easily observed with living cultures than with the dead. A thin bacillary emulsion from a young agar culture may also be employed for this purpose. The preparations are made on a glass slide and covered with a cover-glass. Along with this a control preparation is made with saline only. The edges of the cover slips may be sealed with melted vaseline to prevent evaporation. The preparations are then put in the incubator at 37°C. for a period of $\frac{1}{2}$ to 1 hour, after which they are examined microscopically. In our tropical climate, particularly during the summer time, the preparations may be left on the working table. In a positive case, the bacilli will be found to have collected in clumps, whereas in the control, they will remain free from one another, evenly distributed and actively motile (Fig. 92).

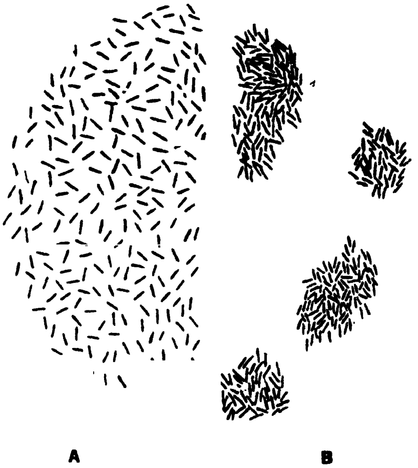


Fig. 92.

Microscopic agglutination reaction.

A– Before the specific serum is added; note the organisms are separate. The same is observed in the control area in which normal saline is added.

B After the specific serum is added; note the clumping of the organisms.

Macroscopic method:—

A. Slide method:—Though it has many fallacies, still it is a very useful and quick method and is often a valuable working guide for determining the identity of a freshly isolated organism. It also enables one to know which of the suspected colonies in a culture is to be submitted for further investigation. A loopful from the suspected colony is rubbed up with saline on a clean glass slide, so as to yield a uniform turbid emu[illegible]. Two such preparations are made and, if it is suspected to be Eberth[illegible] phi, a drop of a diluted antityphoid serum (stock) is added to one [illegible] ulsion and a drop of saline to the other, the latter serving as a contr[illegible] lide is rocked for a minute or so. If the suspected organism is E. typhi, flocculation, as judged by the development of obvious granularity to the naked eye, occurs in the test preparation, whereas the control will remain uniformly turbid (Fig. 93).

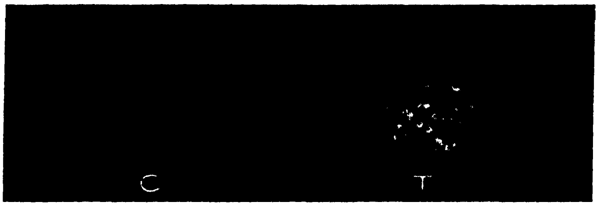


Fig. 93.

Slide agglutination.

C—Control area in which normal saline is added, showing uniform turbidity of the emulsion.

T— Test area showing flocculation or granulation of the emulsion when the *high titre specific serum* is added. The reaction is positive.

B. Dreyer's method:—

MATERIALS REQUIRED:—Vide Fig. 97.

1. Dreyer's rack (Fig. 94). This consists of a metal (copper) rack provided with (a) fifteen small holes in three rows of 5 each, for agglutination tubes, and (b) four larger holes, two at either end, for dilution tubes.

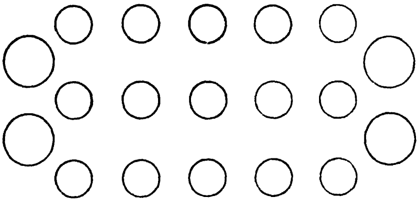


Fig. 94.

Dreyer's rack.

2. Dreyer's agglutination tubes (Fig. 95). These tubes are of 2½″ × ¼″ size with tapering ends. Into these tubes actual agglutination phenomenon takes place.

3. Dreyer's dilution tubes. These are larger tubes of 3″ × ½″ size, in which dilution of the serum is carried out.

4. Dreyer's pipette. This is a special standard pipette by which uniform drops can be measured out.

5. Dreyer's standard water bath. In this a constant temperature of 50° to 56°C. is being maintained by a regulating mechanism, operated by gas (*Vide* Fig. 98) or electricity.

6. Supply of normal saline in a sterile pot.

7. Standard bacillary emulsion *(vide infra).*

8. Patient's serum. The serum must not be hæmolysed, otherwise false positive reaction will appear.

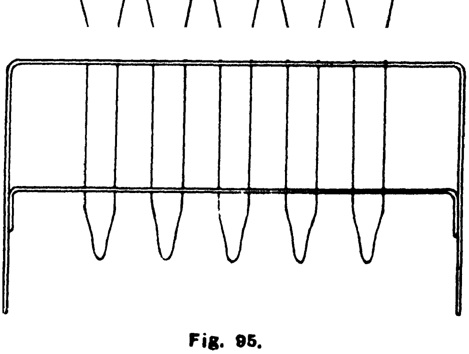


Fig. 95.

Dreyer's agglutination tubes.

HOW TO USE THE TEATED PIPETTE:—A little practice is required to gain a perfect control over the teat. Figure 96 shows the correct method of holding a teated pipette. The pipette is held vertically (so that the drops may be uniform) against the palm by the little and ring fingers. The thumb and the index finger remain free to control the teat. With each change of reagent the pipette is well washed by rinsing it once or twice with boiling distilled water and expelling the moisture as far as possible. Two points are marked with a grease pencil, representing the capacity for 10 drops and 15 drops by volume respectively.

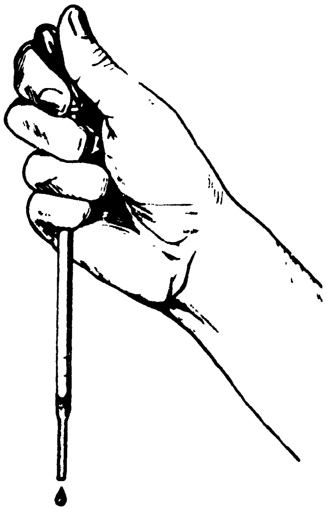


Fig. 96.

Method of holding the Dreyer's pipette during measurement of drops.

FACTORS CONCERNED:—

(i) *Temperature:*—Agglutination reaction is best observed at an optimum temperature of 55°C.

(ii) *Time:*—Time limit, however, varies with different organisms, e.g., for Eberthella typhi, 2 hours and for Eberthella dysenteriæ, an average of 4 hours is necessary. Even in Eberthella typhi, time limit varies, whether H agglutination or O agglutination is estimated. The latter requires a longer time (18 hours) for its reaction.

(iii) *Presence of an electrolyte:*—This is necessary for the agglutination of bacilli and is added in the form of saline.

(iv) *Nature of bacillary emulsion:*—The result will vary if the emulsion is too thin or too thick; hence standard emulsion is necessary. Usually, a strength of 500 to 1,000 millions bacteria per c.c. is found to give the best result. An emulsion of living or dead bacteria may be employed but the latter is obviously preferable owing to the risk of infection, when freely handling virulent pathogenic micro-organisms.

(v) *Gentle motion:*—This greatly facilitates agglutination reaction and is applied in the form of convection currents, by immersing half the length of the tubes into the water bath.

PROCEDURE FOR WIDAL REACTION:—(This is described as used for the diagnosis of typhoid fever).

Test rack:- Fifteen small agglutination tubes and one or two dilution tubes are fitted up in the metal rack (Fig. 97). The patient's serum is diluted 1 in 10 with normal saline in one of the dilution tubes. The actual dilution is carried out by the drop method with the help of the standard Dreyer's pipette. One in ten dilution is obtained by measuring 10 drops of the serum and 90 drops of normal saline and thoroughly mixing them in the dilution tube. This diluted serum is distributed in the tubes as shown in the table below and normal saline is then added so that each tube will contain 10 drops of both combined. The bacillary emulsion is then added, 15 drops

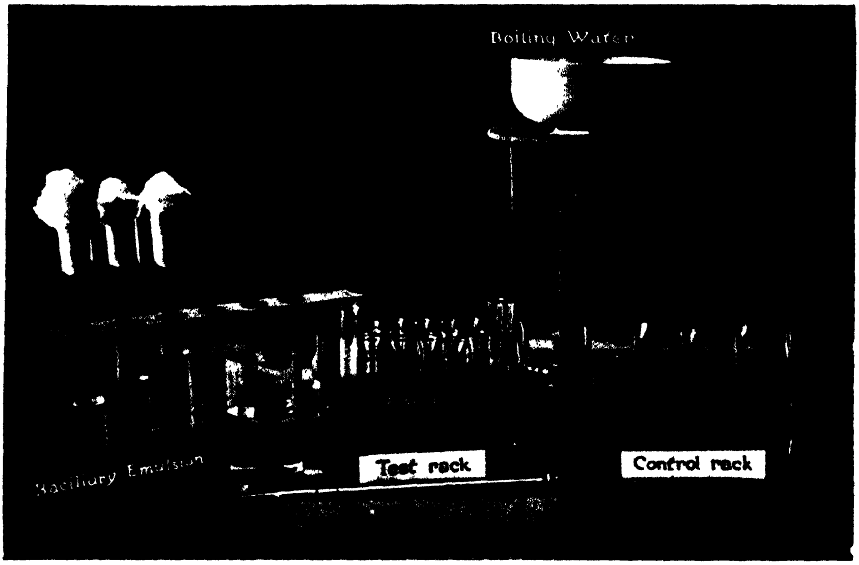


Fig. 97.

Outfit for performing the agglutination reaction by Dreyer's macroscopic method.

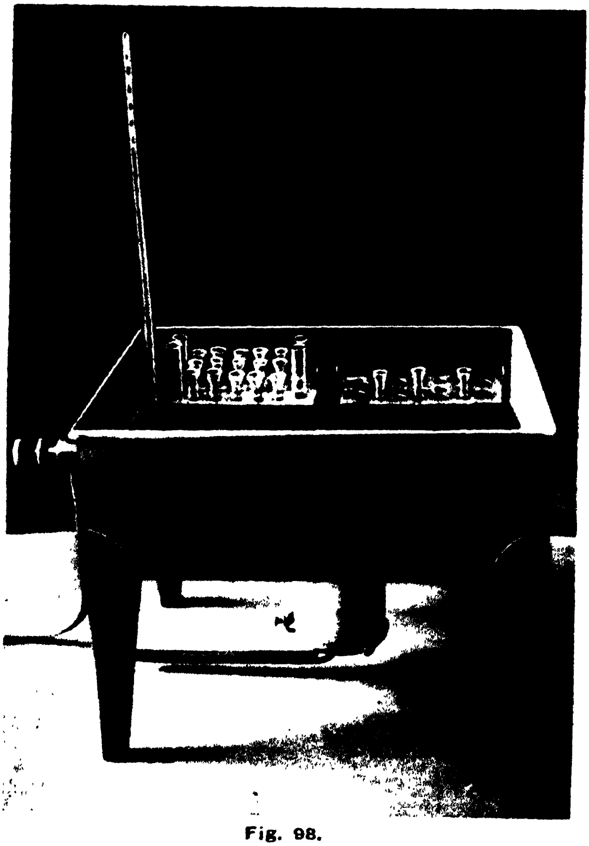


Fig. 98.

Standard water bath in which the test rack and the control rack are immersed.

into each of the tubes, so that the total volume becomes 25 drops in each. Final dilution is then calculated out. While doing the test, a pot of boiling distilled water must be kept going on to rinse the pipette, each time it is used for different reagents. If there is any air-bubble at the botom of the agglutination tube, it should be driven out by gently tapping the tip of the tube. The following table shows clearly the system of dilutions that is adopted in our laboratory.

Number of Tube	I	II	III	IV	V
Drops of diluted (1 : 10) serum	10	5	3	2	1
Drops of normal saline solution	0	5	7	8	9
Drops of standard bacillary emulsion	15	15	15	15	15
Final dilution of the serum	1/25	1/50	1/83⅓	1/125	1/250

When Widal reaction is carried out for typhoid fever, the front row is set up with the bacillary emulsion of Eberthella typhi (B. typhosus), the middle row with Salmonella paratyphi (B. paratyphosus A) and the back row with Salmonella schottmülleri (B. paratyphosus B). The rack is then incubated in a water bath at 55°C., so arranged that the level of water is about half way up the column of fluid in the tubes (Fig. 98).

Control rack (*Vide* Fig. 97): —Along with the test rack, a second one is put up to serve as a control of the bacillary emulsion. For this purpose, 3 agglutination tubes are fitted up in the rack and 10 drops of normal saline are put in each. Then 15 drops of bacillary emulsion of E. typhi, S. paratyphi and S. schottmülleri are added. This control is necessary, because sometimes the bacteria show auto-agglutination. So, in the case of a positive reaction, one must be sure that the emulsion is not agglutinating of itself.

READING THE RESULTS:—After two hours' incubation at 55°C., the rack is removed from the water bath and allowed to stand for 15 minutes at room temperature. Moisture from the tubes is wiped off and the tubes are examined for the formation of clumps visible to the naked eye. These clumps may be floating in the fluid or deposited at the bottom (Fig. 99). If daylight is used for inspection of the tubes, the fingers passed up and down behind them will render the flocculi more visible. The recognition of clumps is made easier when examined against a black back-ground by means of an artificial light.

In a positive case, there will be flocculation, clumping, coarse or fine granulations, according to the strength of the serum. It is better to use a hand lens and a reflected light, so that very fine granulations may not escape detection. The control tube should not show any agglutination. If flocculation appears in the control tube, the result is invalid and the test should be repeated with a different bacillary emulsion. In a negative case, there will be uniform haziness, but no granulations at all.

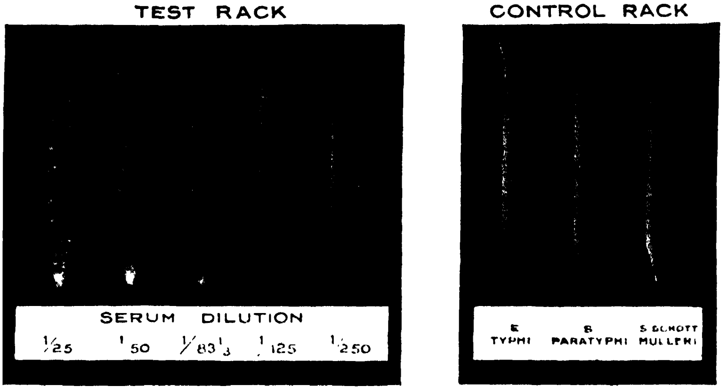


Fig. 99.

Positive agglutination reaction as carried out by Dreyer's macroscopic method. The first three tubes show flocculation and sedimentation and the fourth tube shows only flocculation. There is no change in the fifth tube and in the control rack. The *titre* of the reaction is 1/125.

EXPRESSING THE RESULTS:—Flocculation, and not sedimentation, is taken as the end point, the result being expressed in terms of serum dilution and is known as the *titre* of the serum. Thus, if the end point is observed in tube IV, the titre limit is stated as 1 in 125.

Determination of the titre limit:—If any serum shows positive reaction up to the last tube (1 in 250), the serum has to be further diluted 10 times, i.e., from 1 in 10 to 1 in 100, and the same procedure is to be followed to find out the end point of the reaction. The final dilutions are then 1 : 250, 1 : 500, 1 : 830, 1 : 1250 and 1 : 2500. This determination of the end point is important for diagnostic purposes, because, in the course of an infection with Eberthella typhi, the agglutination reaction, as will be seen later, varies and it is necessary to repeat the reaction several times in the course of the disease. Thus, to compare how the reaction is varying, one must know the titre limit of the serum in all experiments. In some cases, even further dilutions may be necessary and, at each time, it is diluted 10 times more.

Zone phenomenon:—It not infrequently happens that some sera exhibit failure to agglutinate a bacterium in lower dilutions, such as in 1 in 25 to 1 in 50, but the agglutination appears normally in the tubes containing higher dilutions of the serum. This is what is known as the *zone phenomenon*. In fact, the sera which exhibit this inhibition zone often show a very high agglutination titre. It is especially noticed with sera which have been kept for some time or heated at 60° to 70°C. The inhibition of clumping in a zone of lower dilution of the serum may be explained by the following:—

(i) It is partly due to the presence of a non-specific protective colloid which prevents clumping, but is diluted out in higher dilutions.

(ii) It may also be partly due to the alteration of agglutinins into agglutinoid bodies which, as already stated, have greater affinities for the bacteria.

Agglutinoids do not promote agglutination and in stronger concentrations, i.e., in lower dilutions, they prevent the agglutinins to combine with the bacteria. But in higher dilutions, when they are sufficiently diluted, the latter (agglutinins) are effective (Shibley, 1929).

Estimation of O titre:—The H titre of the serum is measured in the manner described above. To estimate the O titre, a special O antigen, i.e., alcoholised or heated (100°C.) suspension, is used and the test is carried out in exactly the same way with the following exceptions. The incubation is prolonged for 4 hours, preferably at 50°C., and the readings are taken on the following day, after keeping the tubes at room temperature overnight. This is necessary as O agglutination occurs slowly. For reading the result, a hand lens should be used, as agglutination occurs in fine granules. In this connection, it should be noted that Felix was able to obtain a strain of E. typhi, known as Felix's strain 901, which is extremely sensitive to O agglutinin and remains so in subculture. Standard emulsion of such a strain can be obtained from the Oxford laboratory.

Dreyer's method is also applicable for the reverse operation, i.e., the identification of an unknown organism with a known serum having a high agglutinating value for that particular organism. This constitutes what is known as the Bordet-Durham reaction. For this test, the suspected colony is subcultured on an agar slope. Twelve to twenty-four hours' growth is taken and is emulsified in 1 per cent. phenol in normal saline, to make a suspension of about 1,500 million organisms per cubic centimetre, as judged by the opacity method. This emulsion is then tested with the stock agglutinating serum and exactly the same technique, as described for Widal reaction, is followed.

SOME SPECIAL TECHNIQUES

Collection of blood serum for Widal's test:—

This is obtained by the following methods:—

1. *By vein puncture:*—About 2 c.c. of blood are withdrawn aseptically with a sterile record syringe, usually from the median cubital vein. The specimen is placed in a sterile test tube and allowed to coagulate in a slanting position. After sometime, the clot contracts and the serum separates out, which may then be removed with a pipette. The contraction may be hastened either by rinsing the syringe or the test tube with normal saline and keeping the blood in an incubator. The separation of clear serum may also be hastened by contrifugalisation.

A very convenient method of collecting blood and sending it to distant places for examination is by *Behring's venule* (Fig. 100 and Fig. 101). This is a small glass capsule from which air has been evacuated, and sealed with a rubber stopper. A fine glass tube passes through the stopper, the inner end of which is so arranged as to remain in [illegible] with the rubber stopper. In this way it acts as a valve and prevents the [illegible] of air. To its outer end is attached a needle which is protected with a [illegible] glass tube, to be filed off before use. The whole tube is sterile. The needle is introduced into the vein and the tube is bent so as to relieve the valve action and blood rushes into the capsule by suction. After filling, it is sealed.

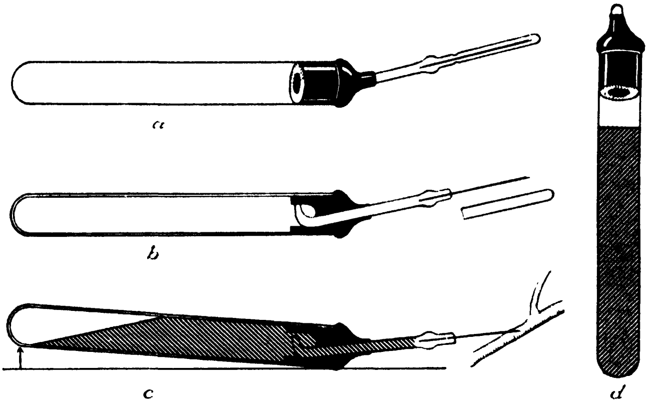
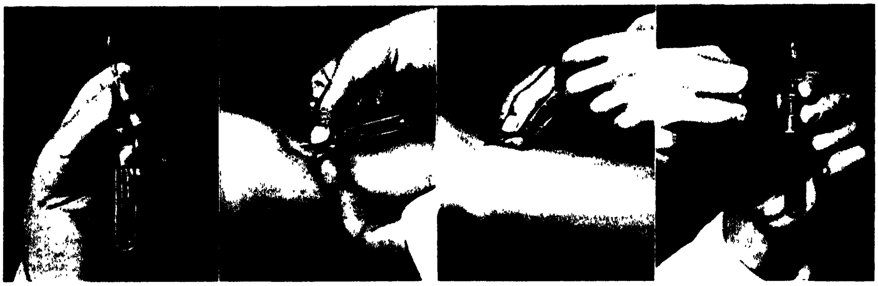


Fig. 100.

Behring's venule and the method of collecting blood.
a, before use; b, ready for use (sectional view); c, valve opened in use (sectional view); d, venule ready for despatch.



1. The glass tube is scratched and sterile needle is now exposed.

The "Venule" should be held thus firmly while exposing the needle and performing the puncture, otherwise it will open prematurely.

2. Thus is the puncture made into the well engorged vein. When the needle is in the vein, blood appears in the glass tube carrying the needle (indicator tube).

3. The valve is opened thus (until accustomed to its use both hands may be used).

4. The indicator tube is filed off and the "Venule" is now ready for despatch.

Fig. 101.

Method of taking blood with Behring's venule (by kind permission of Bayer-Meister Lucius).

2. *By skin puncture:*—In this method, the blood is collected in a Wright's capsule which consists of a plain glass ampoule drawn out at the ends into capillary dimensions and sealed. Before use, both the tips are broken, notching first with a file. One of the capillary limbs is bent in the form of a semicircle, the broken end of which is dipped into the collection of blood. The blood passes into the capsule by capillary action (Fig. 102). The success of the method depends on getting a continuous free flow of blood. To ensure this, a band may be applied round the base of the finger in the form of a tourniquet (great toe in case of infants) and the skin is punctured

near the nail bed by a deep thrust of the needle. The same may be obtained by pressing and squeezing the finger above the point of the needle prick. The needle and the patient's finger are previously sterilised with rectified spirit. When the capsule is three quarters full, the ends are sealed off by heating them in the flame, the straight end being closed first. When this end is cooled, the bent limb may be sealed off without charring the blood. In case of insufficiency of blood, a second capsule may be filled in the same manner. The blood coagulates in the capsule and the serum is allowed to separate, if necessary, by centrifugalisation, using the bent limb for the purpose of hanging. The capsule is opened by filing and breaking the bent-end and the serum is removed by means of a teated capillary pipette.

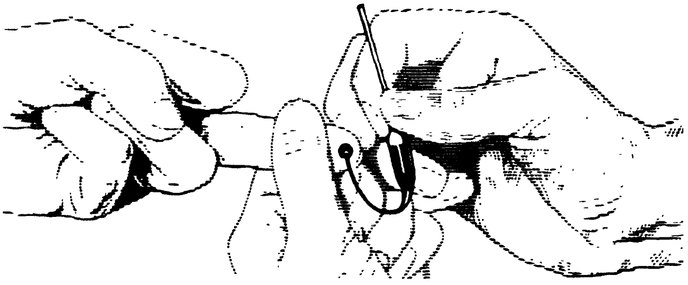


Fig. 102.

Method of collecting blood in a Wright's capsule.

This is a very useful method for transmitting blood from distant places and also for obtaining blood from infants, where vein puncture may not be convenient.

Preparation of standard bacillary emulsion for agglutination reaction. (Description is given for the enteric group of organisms):—

The bacteria, say Eberthella typhi, are subcultured daily from broth to broth, for a period of 15 days and from the last broth subculture, the material is plated on a MacConkey's medium, to see if there is any contamination during the procedures. A suitable *smooth* colony is selected from the plate culture and from a portion of the selected colony, an emulsion is made with normal saline on a clean glass slide. The emulsion is then tested for agglutination reaction with a stock high titre anti-typhoid serum. If flocculation appears, it indicates that the bacteria have not undergone any alteration during subcultures. A 100 c.c. flask of nutrient broth is then inoculated with the remaining portion of the selected colony and kept in the incubator overnight. It is taken out the following day and formalin is added to the emulsion, in strength of 0·1 per cent., the mixture being shaken vigorously and repeatedly. The flask is then preserved in the refrigerator for 4 days, vigorous shaking being applied eac [illegible] for several times. After 4 days' cold storage, the flask is taken out a [illegible] ntrol is made by inoculating a broth tube with the bacillary emulsion [illegible] he flask, to see whether the bacteria have been killed. If there is n [illegible] (sterile), the emulsion is kept in the cold storage or even at the room temperature for a month sealing the mouth of the flask with paraffin to prevent evaporation. This process is required because, when the organisms are freshly prepared, they may exhibit auto-agglutination, but if they are kept for some time, this defect is overcome. Finally, the emulsion is standardised, so as to contain 500 to 1,000 million organisms per c.c., as judged by comparing with the opacity tubes; the dilution being carried out with 0·1 per cent. formol-saline.

A similar procedure is adopted for other organisms of the enteric group. When all the emulsions are ready, they are tested for cross-agglutination before they can be finally used. For this, three stock antisera of E. typhi, S. paratyphi and S. schottmülleri are required and on a clean glass slide, each of the bacillary emulsions is tested for agglutination reaction with the three antisera in succession. It will be observed that flocculation should appear only with its specific antiserum, i.e., emulsion of E. typhi should be agglutinated only by anti-typhoid serum but not by the others. Similarly, the emulsion of S. paratyphi and S. schottmülleri should be agglutinated only by their specific antisera.

Preparation of special O antigens:—This is prepared as follows: —

An eighteen to twenty-four hours' growth on agar is taken and it is necessary that it be of the smooth variety and devoid of R elements, as far as possible. The culture is washed off, preferably with distilled water, and a dense suspension is made, so that it contains 3,500 to 5,500 millions of organisms per c.c. The emulsion is then placed in a sterile measuring cylinder and half its volume of absolute alcohol is slowly added, the mixture being stirred continuously. The cylinder is plugged and allowed to stand for 24 hours at 37°C. The supernatant fluid is decanted off into a bottle and forms the stock O suspension. It is kept without any preservative, such as formalin or phenol, as it has been found that the latter usually inhibit somatic agglutination to some degree. Before use, it is diluted 1:6 by adding 5 parts of distilled water and thereby reducing the concentration of the alcohol sufficiently to prevent any interference with the test. Another method of inhibiting the production of flagella and the corresponding heat-labile antigen is to cultivate the organism in a nutrient agar to which 0·1 per cent. phenol has been added. In this way, H antigens are eliminated and non-motile O forms are produced naturally. But the change from H to O forms is not abrupt and there are colonies of intermediate antigenic constitution. Owing to this uncertainty, the artificial O antigens are commonly used. In order to exclude the intrusion of these intermediate forms (H antigens), it is better to heat the suspension or to treat it with alcohol in the way as already described and thus ensure a double safe guard.

Preparation of agglutinating sera:—For the identification of organisms by agglutination reaction, antisera of high titre are employed. These are obtained by immunising animals with living cultures. In the laboratory, the animal employed for the purpose is the *rabbit*. Before giving the injections, the serum of the animal should be tested for its agglutinating property with the organism that is to be introduced. It is supposed not to produce any reaction. Three to five intravenous injections (intraperitoneal or subcutaneous injections may also be given) of 24 hours' broth culture of a virulent strain of the organism (say Eberthella typhi) are then given at intervals of 7 days. The initial dose is about 0·01 c.c. and the successive doses are given at weekly intervals as follows: 0·1 c.c., 0·5 c.c. and 1 c.c. Seven to ten days after the last injection, a specimen of blood is withdrawn from the ear vein and its titre limit is determined. If it is high, i.e., 1 in 20,000 or more, the animal is bled; about 20 c.c. of the blood are taken, either from the heart or from the carotid artery. The blood is received in a sterile test tube or flask and the serum is separated after allowing it to stand for 24 hours. The serum is pipetted off and distributed in 1 c.c. glass ampoules, sealed and preserved in the ice-chest and, in this way, its titre value can be maintained without any marked deterioration for a period of 4 to 5 years. Since somatic agglutination is often interfered with by the addition of phenol, it is best to preserve it sterile and untreated, or mixed with an equal quantity of glycerin. The same technique is adopted for other organisms, but when Shiga's and Flexner's dysentery bacilli are used, they should be diluted 100 times further. The various agglutinating sera that are in common use may be bought from the market.

HÆMAGGLUTININS

The phenomenon of agglutination is not peculiar to bacteria. Agglutinins can also be produced against red blood cells by injecting them into an animal of a different species, the hæmagglutinins so developed having a corresponding specificity. When such hæmagglutination is observed among individuals of the same species, they are called iso-hæmagglutinins or iso-agglutinins. The discovery of the existence of such specific iso-hæmagglutinins among human beings and also of iso-hæmolysins to be described later, necessitates the utmost care for the proper selection of a donor for blood transfusion. These iso-hæmagglutinins occur naturally, and there exist two agglutinins, *α* and *β*, with two agglutinogens, A and B, among human beings, the various combinations of which have rendered possible to divide them into 4 blood groups. For a detailed study of these hæmagglutinins, special references should be consulted.

PRECIPITINS

The discovery of precipitins was first noted by Kraus in 1897. These substances are closely related to, if not identical with, agglutinins. If a clear extract (filtered culture) of bacteria is added to a specific antiserum, a precipitate occurs. The substance present in the bacteria-free filtrate is called precipitinogen and the reacting substance present in the serum is called the precipitin. The phenomenon of precipitation reaction is intimately related to agglutination reaction, the only difference being that in the former the antigen is used in the soluble state, whereas in the latter, it is in suspension. Like agglutinins, precipitins are specific antibodies and belong to Ehrlich's receptors of the second order. Like agglutinins, they also play a certain part in the problems of immunity and, [illegible] precipitating the infecting agent, favour its phagocytosis.

Practical application of the precipitation reaction

I. *In bacteriology:—*

1. For the diagnosis of certain diseases such as cerebro-spinal fever and syphilis (Kahn's reaction).

2. For the detection of the various types of Diplococcus pneumoniæ.

II. *In medico-legal practice:—*

1. For the detection of human blood in stains on clothings and weapons.

2. For the detection of fraud in the manufacture, e.g., substitution of one kind of meat for another.

III. *To zoologists,* it provides an assistance in disputed questions of animal relationship.

CHAPTER XV

COMPLEMENT AND IMMUNE BODIES

COMPLEMENT:—This is often referred to as "alexin," but is not identical with Buchner's "alexin" which indicates bactericidal substance of the normal serum (*vide* p. 114). The term complement was given by Ehrlich to a substance which is present in normal serum and is necessary for the union of antigen and its corresponding immune body. It has, however, no affinity for antigen when the specific immune body is absent. The complement unites with the antigen-antibody complex and produces lysis or other actions, depending on the nature of the immune body. The action of the complement is best manifested at 37°C. and is without any action at 0°C. The union of the complement with the antigen-antibody complex is a firm one and *not at all reversible*. It is to be remembered that complement is not an antibody and cannot be increased by immunisation; it is entirely non-specific, being available at all times for any immune body and *from the serum of any animal*. The amount of complement, however, varies in different species of animals, being present in large amounts in the guinea-pig's serum. This substance is thermolabile, i.e., destroyed by heating at 55°C. for half an hour. Hence, a serum which has been heated to the above temperature is said to be *inactivated*, as it is devoid of any complementary action.

IMMUNE BODIES:—Substances which develop in the serum due to the action of an antigen and which require for their demonstration the presence of a complement, are designated by the generic name *immune bodies* or *intermediate bodies* (R. Pfeiffer). These immune bodies are named according to the nature of the effects which they produce.

(i) When they involve the killing of bacteria without any actual lysis, they are referred to as *bactericidins*.

(ii) When they lead to the lysis of bacteria, erythrocytes and other cells, they are called bacteriolysins, hæmolysins and cytolysins respectively, or simply as *lytic bodies*.

(iii) When by their action, there results a disappearance of the complement from a mixture of antigen, its specific immune body and complement, without causing any actual lysis of the antigen, they are called *complement-fixing immune bodies*.

(iv) When the immune body, in co-operation with the complement, leads to increased phagocytosis, the term *opsonic immune body is used*. This is particularly observed with antipneumococcal [illegible] which produces its beneficial effect, not by extracellular but by intracellular lysis of the bacteria within the phagocytes.

Thus, the above terms are conveniently employed to indicate the property of any immune body in question. These immune bodies are developed as a result of progressive inoculation by living, attenuated or killed microbes, as well as, by any other foreign proteins. The immune sera so developed are called *antibacterial sera*. Such immune bodies are not produced in animals immediately upon inoculation, but take some time before they can be recognised in the blood. Once developed, however, they persist for a long time and even if they disappear from the blood serum, a slight stimulus, produced by the infection of the specific organism or introduction of a homologous protein, will result in their reappearance in abundance in the inoculated animal. When the immune body combines with its specific antigen, the latter is said to be sensitised; hence Bordet referred to an immune body as a *sensitiser* or a *substance sensibilisatrice*, a name which is still used by the French workers. The union between an antigen and its specific immune body takes place best at 37°C., but may also occur at a low temperature (just over 0°C.). Once united, the combination is fairly stable. Of the other properties of immune bodies, the following are the two most important ones:—

(i) *Specificity*, i.e., they are elaborated only in response to a particular antigen and will act on that antigen only.

(ii) *Thermostability*, i.e., they are resistant to heating at 55°C., but may be destroyed at 70°C.

Unity or multiplicity of complement:—A keen controversy was held for several years and enquiries were carried out to determine whether the complement was one single substance or whether there were many different complements for different kinds of immune bodies. There are two rival views. On one side, Ehrlich, assisted by Morgenroth, favours the view of multiplicity of complement and on the other side, Bordet maintains the views of unity of complement. The data so far available points to the unity of complement, i.e., one and the same kind of complement is concerned in the various actions of the immune bodies.

On the nature of the union of complement and immune body:— Here again there are two views, one advocated by Ehrlich and the other by Bordet.

EHRLICH'S VIEW (Fig. 103):—He considers these immune bodies as his receptors of the third order having two combining arms. The one which combines with the complement is called the *complementophilic arm* and the other which combines with bacteria or other cells, i.e., antigen-containing cell, is called the *cytophilic arm*. It is for this reason that the name *amboceptor* was given by Ehrlich (*Ambo*—both; *capio*—I seize). Complement, according to him, may be represented as possessing a haptophore group which enables it to combine with one arm of the immune body (amboceptor) and a zymophore group by which it produces its toxic action on the antigen. Thus, Ehrlich considers that the immune body acts as a connecting link between its specific antigen and the complement and it is through this intermediary that the complement can exert its toxic action.

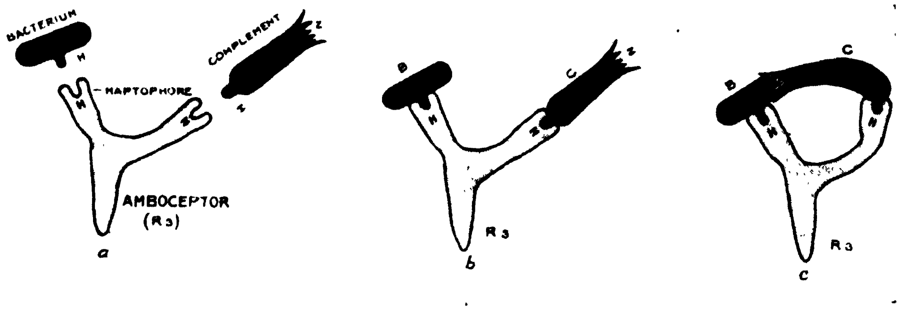


Fig. 103.

Ehrlich's conception of the union of the amboceptor and complement with the antigen. (A diagrammatic representation.)

BORDET'S VIEW (Fig. 104):—Bordet however denies that the complement ever unites with the immune body as such. He regards that the union between the immune body and its specific antigen is an adsorption process, resulting in some alteration of the physical conditions of the surface. When the antigen has combined with its specific antibody, it is said to be sensitised and hence, the immune body is referred by him as a *sensitiser* or *substance sensibilisatrice*. The complement, in his view, does not unite with the sensitiser but acts directly on the antigen which has been altered by union with the immune body.

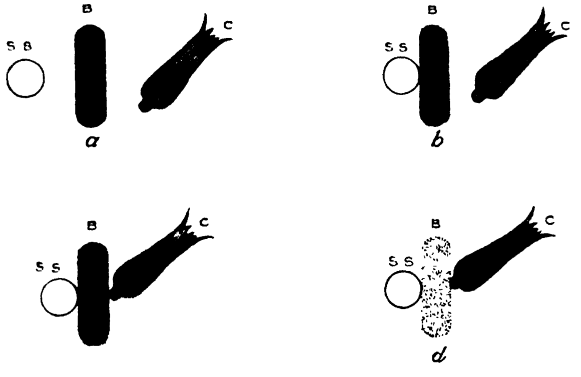


Fig. 104.

Bordet's view about the union of complement (c), substance sensibilisatrice (S.S.) and the antigen (bacterium B): a. three substances are separate; b. sensitisation of the bacterium, c. union of complement with the sensitised bacterium; d. lysis of bacteria. (A diagrammatic representation.)

Nature of complement:—The chemical structure of the complement is not definitely known but there is evidence to show that it is a complex body and can be split up into several fractions, all of which are responsible for the complementary action.

Deviation of complement *(Neisser-Wechsberg phenomenon)*:—Neisser and Wechsberg (1901) showed that in [illegible]icidal experiments with a suspension of bacteria, immune body and [illegible]ment, a great excess of the immune body hinders rather than helps [illegible]dal action. It may be regarded as a zone phenomenon, analogous to what is seen in the agglutination reaction.

Fixation or absorption of complement *(Bordet-Gengou phenomenon)*:—Bordet and Gengou, in 1901, demonstrated that many antigens, in the presence of their specific immune bodies, are able to remove the complementing property from sera *in vitro*. Such alteration in the quality of a serum has been ascribed to the using up of the complement by the antigen-antibody complex. The complement is thus said to be absorbed or fixed to the sensitised antigen. As the fixation or absorption of the complement cannot be detected by the naked eye, it has been found necessary to use an indicator in the form of sensitised red cells (red cell and hæmolytic immune body). The experiment is carried out as follows:—

An emulsion of Pasteurella pestis (antigen) is mixed with antiplague serum (specific immune body) which has been heated to 55°C. for half an hour to destroy its complement. To such a mixture, complement is added. After incubation at 37°C. for one hour, the indicator, i.e., either the sensitised red cells or the red cells and inactivated hæmolytic serum, is added and the whole series again incubated for 1 hour at 37°C. In this case, no hæmolysis will take place showing that all the complement has been used up by the bacterium-sensitiser complex and none is left free to combine with the sensitised red cells to cause hæmolysis (Fig. 105A). This experiment proves that when the antigen and antibody are homologous, the complement

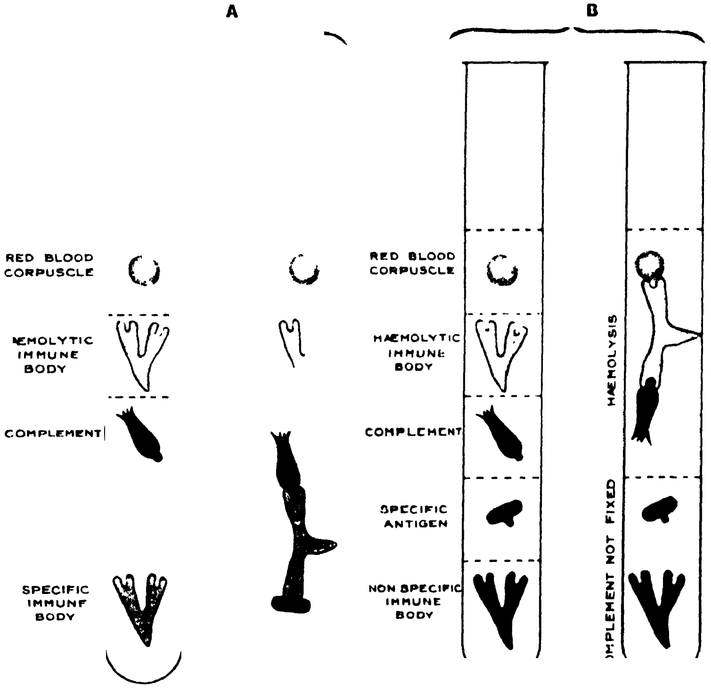


Fig. 105.
Diagrammatic representation of the complement-fixation test, in the Bordet-Gengou reaction, as conceived by Ehrlich.
A, fixation of complement in the presence of specific antigen and antibody; B, complement is free showing that antigen and antibody are non-specific.

can be fixed. The converse of this can be shown by using either another organism, say cholera vibrio, instead of P. pestis, or another serum, say anti-typhoid serum, instead of anti-plague serum. In both these cases, complement remains free and is able to combine with the sensitised red cells to cause hæmolysis (Fig. 105B). Thus the occurrence of hæmolysis will demonstrate that the antigen and antibody are not homologous and specific. This may be summarised in the following table:—

	A.	B.	C.
Antigen	Emulsion of P. pestis	Emulsion of Vibrio comma	Emulsion of P. pestis
Immune body	Plague immune serum heated to 55°C.	Plague immune serum heated to 55°C.	Antityphoid serum heated to 55°C.
Complement	Fresh normal serum	Fresh normal serum	Fresh normal serum
	Incubated for 1 hour at 37°C.		
Result	Complement fixed	Complement free	Complement free
Indicator (sensitised red cells) is now added to A, B and C and all are incubated at 37°C.			
Final result	No hæmolysis	Hæmolysis	Hæmolysis

USE OF THE COMPLEMENT FIXATION TEST:—

1. To determine the presence of the antigen when the specific antibody is known.

2. To determine the presence of the antibody when the specific antigen is known. This is the rationale of *Wassermann's reaction.*

Lytic phenomena:—To demonstrate the phenomenon of lysis, three things are necessary, such as (i) *Antigen,* (ii) *Specific antibody* and (iii) *Complement.* Of the various lytic phenomena, the two most important ones are bacteriolysis and hæmolysis.

Pfeiffer's reaction:—This is a phenomenon of bacteriolysis in *vivo* and is chiefly used for the identification of cholera vibrio. The reaction can be demonstrated in two ways:—

(a) A guinea-pig is first immunised with successive inoculations of highly virulent cholera vibrio, after which it receives into its peritoneal cavity 5 to 10 times the ordinary fatal dose of an emulsion of cholera vibrio.

or (b) A like dose of cholera vibrio mixed with an excess of anticholera serum obtained from an immunised rabbit is injected intraperitoneally into a normal guinea-pig.

In either case, from time to time some of the peritoneal fluid is removed with a pipette through a skin incision and [illegible] under a microscope. Pfeiffer noted striking morphological chang[illegible]ticularly swelling and granular degeneration of bacteria with their [illegible]ed disintegration. The injected bacteria become gradually smaller an[illegible]ler and finally undergo solution, like a lump of sugar in water, as de[illegible] by Pfeiffer.

CHAPTER XVI

HYPERSENSITIVENESS

(Anaphylaxis, allergy, idiosyncrasy and serum sickness.)

This term is applied to the condition of increased susceptibility of animals to substances of the nature of foreign proteins introduced into their bodies. Such hypersensitiveness may be inherited or acquired by bacterial infection, as well as, by parenteral introduction of foreign proteins. Thus, the condition may be discussed as follows:—

I. ***Induced or acquired***—Under this group are included anaphylaxis and allergy.

(a) **Anaphylaxis**—This term is applied to a condition of hypersensitiveness which is induced by the *previous introduction of a foreign protein.* This may again be general and local.

(1) *GENERAL ANAPHYLAXIS*, which may be (i) *active* and (ii) *passive*.

(2) LOCAL ANAPHYLAXIS OR ARTHUS PHENOMENON.

(b) **Allergy—**This term is applied to the condition of hypersensitiveness which is induced by *bacterial infection.*

II. ***Natural—***Under this group are included idiosyncrasies and serum sickness.

(a) **Idiosyncrasy or Atopy** (atopia -- a strange disease)—Such a reaction may express itself chiefly (i) in the respiratory system, e.g., asthma, vasomotor rhinitis, hay fever, recurrent catarrhs; (ii) by skin rashes; (iii) by effusion into joints; and (iv) in the alimentary canal by diarrhœa and vomiting.

(b) **Serum sickness.**

ANAPHYLAXIS

So long it has been discussed that an animal usually becomes more and more tolerant to injections of an antigen which may be toxins or any foreign proteins, resulting in the development of immunity. While such is the usual rule, sometimes, however, the exactly opposite effect is produced and, instead of tolerance, there is increased sensitiveness to it. One may frequently hear about it in his student-life from his respective physicians and surgeons and will himself dread of its occurrence when he will want to administer serum in his professional career, either for prophylaxis or for curative purposes. And if he is not on the alert on this point, sometimes, he may have to suffer.

In actual life, one may have to inject antitoxic sera in a patient who had previously received serum injections for some other reasons. In some of these cases, after giving the injection, either knowingly or unknowingly, dangerous and very alarming symptoms develop. These symptoms are very sudden in onset and may be manifested by extreme distress, dyspnœa, cyanosis, œdema, collapse, respiratory failure and sometimes, even convulsion. Death may follow very rapidly. The same thing had been noticed during active immunisation of animals against diphtheria and tetanus toxins, in the earlier days of immunology. To such a condition, the term *anaphylaxis* was applied by Richet in 1902 (*ana*, without; *phylaxis*, protection), as opposed to prophylaxis. It should, however, be remembered that sudden death after the injection of an anti-serum is extremely rare in actual practice, although reactions of various grades and intensity may occur after such injections.

Sensitisation of animals to foreign proteins varies greatly. The guinea-pig, horse and goat are readily sensitised; the rabbit, dog and probably man are very slightly susceptible, while the mouse is still less so, and the rat is almost completely refractile. It is for these reasons that experiments on anaphylaxis are mostly carried out on *guinea-pigs*.

Mechanism:—For an anaphylactic shock to appear in an animal, the latter must be previously *sensitised*. This is usually done, in practice, by injecting some foreign protein into the tissues of the animal. Then a second injection of the same protein, after 10 days, will produce the typical symptoms which will vary with different species of animals employed for the experiment. Thus, in a guinea-pig, the most marked effect is on the respiration which becomes embarassed and slowed and the animal may die of asphyxia, a condition which results from the spasm of the bronchial muscles. In rabbits and dogs, there is absence of dyspnœa, the outstanding feature being a marked fall of blood pressure. Although these symptoms may vary from animal to animal, they are remarkably constant for a particular animal, no matter by what antigen the condition is produced. Horse serum, milk, egg albumen or other antigens, when injected into a guinea-pig, will cause precisely the same symptoms, but their effect on the rabbit and dog will be entirely different. This is due to the varying susceptibility of different tissues in different animals.

The foreign protein or similar body (antigen) which, primarily injected into an animal, makes it sensitised to it, is called the *sensitising dose*, and the antigen is called the *anaphylactogen*. This is usually a soluble protein and, by itself, is non-toxic to the animal. Usually about 7 to 10 days must elapse before the animal is fully sensitised. If a second injection is made during this period, no untoward effect is produced. This period, during which further injection of the antigen does not produce any effect, is called the *incubation period*. If, after the lapse of this period (10 days), a second injection is given, symptoms of anaphylactic shock may be produced. The injected material which gives rise to the production of the anaphylactic shock is known as the *assaulting dose, the toxogenic* [illegible] or *the reacting dose.*

Sensitisation:—

QUALITATIVE: All anaphylactogens are good antigens which can be weakened or destroyed by the same physical, chemical and fermenting reagents as any other bacterial antigens. They must be foreign to the circulating blood of the animal. Globulins and vegetable proteins are good anaphylactogens.

QUANTITATIVE: The amount of protein required to sensitise an animal may be incredibly small. For a guinea-pig, it may be 0·01 to 0·1 c.c. In this connection, it should be noted that larger doses, specially if repeated, protect the animal for weeks from the assaulting dose; this is called the *refractory state.* The serum of the animal, in the refractory state, has an abundant supply of antibodies and is thus suitable for passive sensitisation. When the refractile animal receives the assaulting dose, the latter finds enough antibody in the serum to combine with, and in this way the sensitive tissues are prevented from combining with the anaphylactogens.

TIME FACTOR: With the standard dose of 0·01 to 0·1 c.c. of foreign protein, the guinea-pig begins to be sensitised in 8 days and the maximum is reached in about 3 weeks. Thereafter it declines but remains sufficiently high for six months. Some degree of sensitiveness is probably permanent. Larger sensitising doses prolong the incubation period.

Pathology:—The essential pathological process consists in the *contraction of the plain muscles* and *dilatation of the capillaries.* The changes in the different organs are as follows:—

LUNGS: Owing to a spasmodic contraction of the unstriped muscles of bronchioles, their lumen is narrowed and consequently air cannot escape through the constricted bronchi. For this reason, the organs are found voluminous and distended with air. They are pale in colour, emphysematous and bulge forward over the heart. This condition of the lung is called *acute vesicular emphysema.* The mucous and submucous layers of the bronchioles are considerably swollen owing to marked vaso-motor turgescence and this also adds to a further constriction of the tubes. Thus, the entire change in the lungs is due to *bronchomotor spasm* and *vaso-motor turgescence,* the result of excessive vagal activity.

LIVER: A very important reaction takes place inside the liver. Owing to the spasm of the muscular coat of the hepatic veins, there is considerable obstruction to the flow of blood from inside the liver into the Vena cava. This results in an intense congestion of the liver and a rise in blood pressure in the splanchnic area and a consequent depletion of the systemic circulation. This gives a very characteristic post mortem appearance. The liver is swollen and cyanotic and the splanchnic viscera are all congested, with subserous and submucous hæmorrhages in the intestines.

BLOOD: Owing to the tremendous dilatation of the splanchnic vessels, there is a great fall of blood pressure. Diminution or loss of coagulability, and pronounced leucopenia are the two important blood changes. There may be eosinophilia, a reaction which has been supposed to deal with foreign proteins, whether in the skin, bowels or bronchi.

As already mentioned, these changes vary with different animals in which anaphylactic reaction is produced. Thus in guinea-pigs, the most important change occurs in the lungs, whereas in the rabbit and dog, the chief change is a great accumulation of blood in the venous system with enlargement of the liver.

Desensitisation or anti-anaphylaxis:—This may be produced in the following ways:—

(i) After recovery from an anaphylactic shock.

(ii) By giving the second injection within the latent period, i.e., within 10 days after the sensitising dose.

(iii) Temporarily, by drugs such as atropine and adrenaline.

(iv) By the injection of graduated increasing doses. This is usually done in practice and is carried out as follows:—

Method:—A test for suspected susceptibility should first be made. This may be done by injecting 0·1 c.c. of the serum to be used, *intradermally*, into the patient. If no effect follows within half an hour, the patient is not sensitive. If however, a red, urticarial wheal develops at the site of the inoculation within this period, the patient may be considered sensitive. Desensitisation is then carried out by repeatedly injecting subcutaneously 0·5 to 1 c.c. of the serum and gradually increasing the doses, at intervals of 5 to 10 minutes. In this way, a large amount of serum may be given in a comparatively short period, without producing symptoms of anaphylactic shock.

NOTE: When several injections of serum are to be given to a patient and a long time is likely to be necessary for the completion of the treatment, the anaphylactic symptoms may be avoided by giving the serum injections at the intervals of not more than a week, preferably less, so as to increase the refractory state. This is very important for the treatment of diphtheria and tetanus.

Theories as to the causation of anaphylactic shock:—When an antigen is injected, the sensitising dose appears to produce some antibody (*anaphylactin*), the nature of which has been shown by various workers to correspond closely with precipitins. When the second dose is injected, it combines with the antibody which has been formed as a response to the first injection. As a result of this combination, there develops either a toxic product or some alteration in the tissue elements, which sets up a violent reaction in the cells, giving rise to the production of the anaphylactic shock. The substance which produces the anaphylactic shock is termed *apotoxin* by Richet and *anaphylatoxin* by Friedberger and, according to the modern conception, it is supposed to be *histamine* or a substance very similar to it.

It is, therefore, an expression of an antigen-antibody reaction which bears the character of strict specificity and is demonstrable only in the living tissue. The actual site of reaction may be either in the circulating blood or in the tissues and, accordingly, two chief views have been put forward, the former is called the humoral theory and the latter the cellular theory.

Humoral theory:—

ANAPHYLATOXIN THEORY OF FRIEDBERGER:—The union between the antigen and the antibody in the circulating blood of an animal, is followed by the liberation of a ferment which splits off from it one or more of the poisonous products of protein decomposition. According to him, anaphylatoxin is set free as the result of proteolysis and it is this toxic protein-cleavage product which is responsible for the development of anaphylactic phenomenon. There are many fallacies about this theory and it is very difficult to explain such a widespread change produced by an incredibly small amount of the toxin. Moreover, a ferment action with resulting proteolysis cannot proceed with the explosive rapidity necessary to account for the instantaneous development of the effects.

BORDET'S THEORY:—The union of the antibody and the antigen creates a complex which, by absorption, monopolises certain principles in the blood plasma which then becomes toxic.

Cellular theory:—There are various facts which tend to show that the humoral theory cannot be tenable. The arguments against the humoral theory are:—

(i) An animal may give an anaphylactic reaction when its blood does not contain any antibody, thereby disproving that antigen cannot react without its specific antibody in the circulating blood.

(ii) Blood of an animal in acute anaphylaxis is non-toxic and thus fails to demonstrate the presence of a toxin which is supposed to be formed by the union of the antigen and the antibody in the blood.

(iii) An animal may fail to develop any anaphylactic shock when its blood contains an abundant supply of antibody, as happens in the refractory state of the animal. Had a toxin been formed by the antigen-antibody union in the circulating blood, one would expect a violent reaction. The serum of the refractile animal, however, when injected into a normal animal, passively sensitises the latter. The above facts show very clearly the protective function of the circulating antibody.

(iv) In passive anaphylaxis, the assaulting dose annot produce the reaction immediately but a certain time elapses before the tissues become sensitised to the injected antibodies. During this time, most of the antibody has disappeared from the blood stream. This clearly suggests that it is the *fixed*, and not the *circulating, antibodies* that are concerned in the reaction.

(v) Replacement experiments in animals in which the blood of the sensitised animal is replaced by saline or normal blood, leaves the animal still anaphylactic. This shows that it is the sensitiveness of the tissaes which is responsible for the anaphylactic reaction.

(vi) Specific hypersensitiveness may be demonstrated in plain muscles, such as the intestines (*Schultz*) or the uterus (*Dale*), excised from a sensitised animal and suspended in Ringer's solution.

Thus, all the above facts prove that the antigen-antibody union occurs in or on the cells of the tissues. The question now arises, how the union may bring about the anaphylactic reactions.

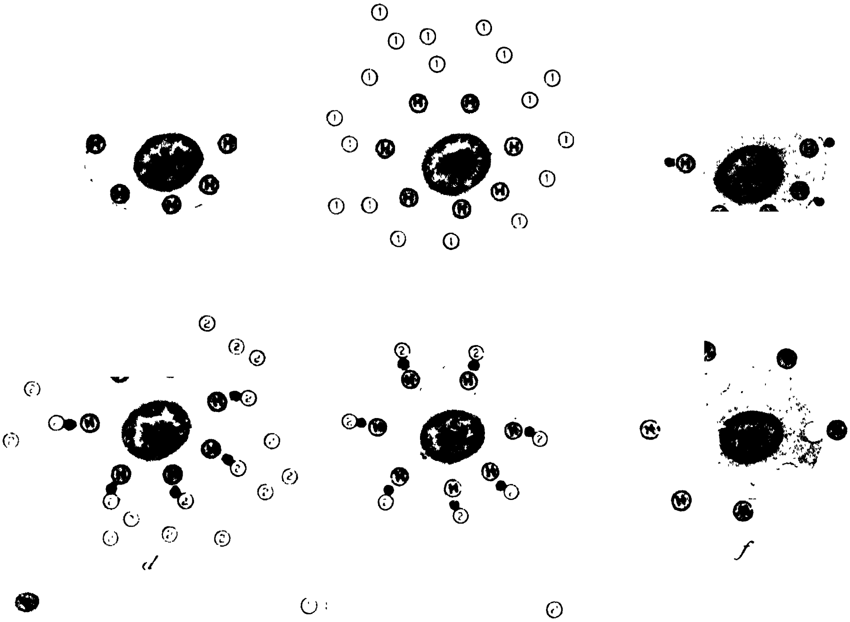


Fig. 106.

Illustrating Lewis's histamine hypothesis of anaphylaxis.

ed substance; b, cell and the sensitising antigen; c, formation of antibody on the cell (sensitisation of the cells); d, sensitised cells and the reacting antigen; e, union f antigen anaphylactogen and antibody (anaphylactin) on the cell damages the st ture as shown by the clearing of areas; f, as a result of such injury, the H substa is liberated into the circulation, producing the anaphylactic shock.

LEWIS'S HISTAMINE HYPOTHESIS (Fig. 106):—Working on the responses of the blood vessels of the human skin, Lewis has brought forward evidences to show that the cause of the capillary reaction in skin irritation is due to the liberation of a *preformed substance* present in the living epidermal cells as the result of tissue injury. This substance which he refers to as the H-substance, is considered to be either histamine or a complex of similar activity and is responsible for the reaction. The normal tissue cells contain this preformed substance in varying amounts, which is discharged into the blood stream whenever there is any tissue injury. The action of this substance will be greatest on those cells which are sensitive to histamine. In anaphylactic reaction, the tissue injury is supposed to be due to the union of the antigen with the antibody inside their substance or on their surface layers. As a result of this, the preformed histamine is liberated into the circulation which acts with the greatest intensity on the histamine-sensitive cells, producing the typical reaction of anaphylactic shock. Unless the antigen-antibody union takes place in the tissu[illegible]re will be no injury and hence no anaphylactic shock. It is in this way [illegible]he refractory state of the animal can be explained, because owing to the [illegible]ce of a large amount of antibody in the circulating blood, the tissue antibodies are prevented from uniting with the antigen and therefore no injury to the tissue cells, no liberation of histamine and no anaphylactic shock occur. (Fig. 107).

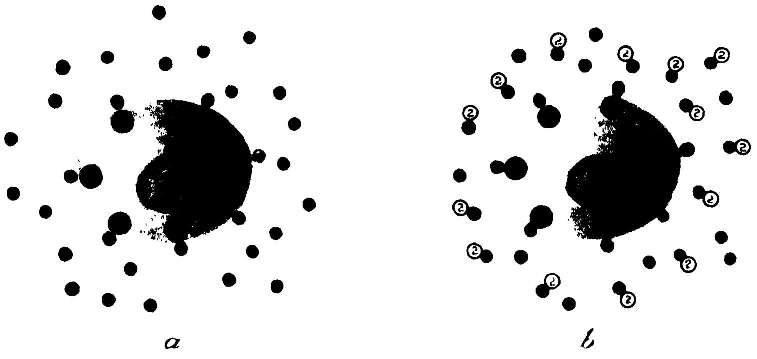


Fig. 107.

Diagram illustrating the refractory state of the animal.
a. shows an excess of antibodies on the cell and in its surrounding fluids;
b. the injection of reacting dose produces no shock because all the antigens are fixed up by the circulating antibodies and none can unite with those on the cell.

Passive anaphylaxis:—This denotes that the acquired susceptibility can be conveyed by injecting the serum of the actively sensitised animal into another normal animal. When the latter gets the assaulting dose the symptoms of anaphylactic shock develop after a latent period of 4 to 24 hours. This condition usually disappears in a few weeks' time, as opposed to active anaphylaxis which persists much longer. It has also been demonstrated that the anaphylactic state can be transferred by an anaphylactic mother to her offsprings and such a sensitiveness may last for some time after birth.

Local anaphylaxis—Arthus phenomenon (1903):—If a rabbit receives several injections of serum at intervals of some days, then another injection causes an œdematous mass, an aseptic abscess or an area of necrosis, at the site of the new inoculation, which may be far removed from the region of the previous injections.

ALLERGY

This denotes a specific inflammatory reaction produced in infected animals by toxins of the infecting bacterium or other parasite, which, in normal animals, are relatively without any effect. It should be definitely noted that the most important criterion for allergy to develop is the *existence of a previous infection, whether latent or cured.* For instance, when a person is perfectly free from tuberculous infection and from which he never suffered at any time in his life, he cannot show any allergic manifestation, if tuberculin is injected into his tissues. On the other hand, if a person had once suffered from a tuberculous infection and his tissues had thus been sensitised to the toxins of the Mycobacterium tuberculosis, the introduction of tuberculin into his body will be attended with allergic reactions. Similarly, only those animals which are actually infected with Pf. mallei will respond to the injection of the toxin prepared from the organism while those which are free from infection, will never show any reaction whatsoever. Hence, mallein reaction is considered to be an allergic phenomenon. Other examples of similar reactions are typhoidin (in typhoid fever), luetin (syphilis) and Casoni's (hydatid disease) reactions.

Pathology of allergic reaction:—In the early stage, there is essentially an acute inflammation, with marked vascular dilatation, œdema and exudation of red blood cells and polymorphonuclear leucocytes. With the progress of the reaction, such inflammation may go on to necrosis and sloughing, followed by rapid healing.

If one compares the lesion of local anaphylaxis with that of allergic reaction, he will find marked differences between the two. Intracutaneous injection of the specific antigen in the sensitised animal produces, in the early stage, a rapidly developing œdema, suggesting a vaso-motor effect. Thus, it seems that allergic reaction is a fierce and frightened attempt on the part of the tissue cells to counteract the injurious effects of the poison of which the former appear to have a previous experience. It is therefore essentially protective in nature. During a primary infection, the toxins of the infecting organism circulate in the system and thereby sensitise every cell of the individual. This sensitisation remains, more or less, as a permanent condition, conferring on the cells an exceedingly large amount of alertness to the same poison. This persists for years, even when the infection is over a long time ago. Possessed with such acute alacrity, the cells act at once when they meet with the same type of poison and the sum total of such a response is manifested as the allergic reaction.

Relation of allergy to immunity:—Although allergy differs definitely from anaphylaxis, it is certainly an expression of immunity. It has been found that animals previously infected with Mycobacterium tuberculosis and showing allergic reaction are more resistant to subsequent infections than normal animals. This is because, along with the allergic manifestation, the infected animal has, *pari passu*, developed some degree of immunity against the infecting agent. In the human beings, when the allergic state is either absent or has disappeared, as often happens after measles, influenza and other debilitating diseases, it is attended with a high degree of susceptibility to tuberculosis.

Although allergy and anaphylaxis are very nearly related to each other, yet one is not synonymous with the other. There are definite and well marked differences between the two. These are as follows:—

(1) The allergic reaction is usually not so severe.

(2) If tuberculin is injected intravenously into a sensitive (tuberculous) guinea-pig, no anaphylactic shock or similar symptoms are produced.

(3) Passive transference of allergic sensitiveness from one individual to another is not successful.

(4) Unlike anaphylaxis, the allergic reac [illegible] not be desensitised.

(5) The intracutaneous reaction to tuberculin is very different, both in character and also in the rate of development, from the immediate skin reaction seen in local anaphylaxis. The former is due to an inflammatory reaction whilst the latter is due to local vasomotor disturbances.

IDIOSYNCRASY

It has been remarked by Morley Roberts that "immunity is assimilation." To some foreign proteins, the cells of our body are naturally immune, i.e., our body cells can assimilate them automatically; to others, the cells acquire immunity, i.e., they learn to assimilate them. But to some foreign proteins, immunity is neither congenital nor acquired and, as the tissue cells cannot assimilate them, they continue to resent their presence. Such are the substances which are responsible for exciting idiosyncrasy in varying degrees. It has been mentioned by some that it is the last stand of the race against adulteration of its protoplasm. The salient feature of the idiosyncrasy may be regarded as a fierce and frightened attempt on the part of the cell to preserve its chemical identity (Langdon Brown).

The characteristic symptom-complex of idiosyncrasies, which is developed in the individual may be manifested in the various systems as already mentioned. The reacting substances gain access to the tissues by some particular route, either by inhalation or by ingestion, upon which depends the manifestation. The substances which evoke this response may be of a varied nature, such as various food materials (food idiosyncrasies), chemical compounds (drug idiosyncrasies), pollens, horse dander, cutaneous debris of other animals and so on. In this connection, it should be remembered that these idiosyncrasies are expressions of excessive activity of the parasympathetic system (vagal activity) with a relative insufficiency of the sympathetic system, along which there is also a certain amount of endocrine imbalance. Drugs, such as atropine, which paralyse the parasympathetic system and others, such as adrenaline and ephedrine, which stimulate the sympathetic system, are known to produce beneficial results in these conditions.

SERUM SICKNESS

This type of hypersensitiveness is observed in some persons after the therapeutic injection of antisera. In most cases it appears after the primary injection and in this way it differs from anaphylaxis. The symptoms usually develop after an incubation period of 8 to 12 days and consist of a mild pyrexia, urticarial rash, swelling in the joints, albuminuria with or without œdema and enlargement of the neighbouring lymph glands. The condition is variable in its duration and disappears completely. Fatal results are very rare. The reaction is of greater incidence in those individuals who receive a large dose of the serum intravenously than in those who receive a small dose or by some other routes. It is for these reasons that the use of concentrated sera has greatly reduced its incidence. Though serum sickness differs from anaphylaxis, the mechanism is more or less the same. Foreign protein that has been injected remains in the circulation for a much longer period and thus reacts with the antibody it has evoked, producing the characteristic reaction. Thus, it appears to be in close relation with other antigen-antibody reactions and so with anaphylaxis.

CHAPTER XVII

SCHEME TO BE FOLLOWED IN THE STUDY OF AN INDIVIDUAL BACTERIUM

Historical:

Habitat:

Morphology:—

Shape.
Size.
Arrangement.
Capsules.
Spores.
Flagella.
Motility.
Pleomorphism.
Involution forms.

Staining reactions :—

Ordinary aniline stains.
Gram's stain.
Acid-fast stain.
Special stains—for capsules, spores, flagella and metachromatic granules.

Cultural characters:—

Biology Temperature & oxygen requirements.
Reaction of the medium.

Cultivation—Ordinary media—Agar, Broth.
Special media.

Biochemical reactions:—

Sugar reactions, Litmus milk, Indol formation, Gelatin liquefaction, etc.
Metabolic activities—Haemolysis, pigment production, power of reduction, etc.

Toxin formation:

Serological reactions:—

Agglutination reaction.
Precipitin reaction.
Complement-fixation test.

Varieties:

Animal inoculation:—

Susceptible laboratory animals and the characteristic reaction produced.

Pathogenicity:—

In animals.

In human beings.

Source of the organism—Carrier.
Mode of infection & Portal of entry
Pathogenic lesions.

Therapeutic consideration:—

Specific measures—

Passive immunity—Serum, Bacteriophage.
Active immunity—Vaccine, Antivirus, Bilivaccine.

Non-specific measures—

Non-specific protein therapy.
Chemotherapy.

Prophylaxis:

Isolation of the organism:

Laboratory diagnosis:—

Collection of materials.

Methods of diagnosis.

PYOGENIC BACTERIA

These are micro-organisms which are capable of causing suppuration. For practical purposes, these may be classified into two main groups.

I. ***Higher bacteria (Chlamydobacteria):—***

Actinomyces bovis.
Actinomyces maduræ.

II. ***Lower bacteria:—***

A. COCCI.

Gram-positive cocci.

- Staphylococcus.
- Streptococcus.
- Diplococcus pneumoniæ (Pneumococcus).
- Gaffkya tetragena (M. tetragenus).

Gram-negative cocci.

- Neisseria gonorrhœæ (Gonococcus).
- Neisseria meningitidis (Meningococcus).
- Neisseria catarrhalis (M. catarrhalis).

B. BACILLI.

Gram-positive bacilli.

- Corynebacterium acnes (B. acne).

Gram-negative bacilli.

- Escherichia group (Colon group of bacilli).
- Eberthella typhi (B. typhosus).
- Pfeifferella mallei (B. mallei).
- Klebsiella pneumoniæ (Friedländer's pneumobacillus).
- Pseudomonas æruginosa (B. pyocyaneus).
- Proteus vulgaris (B. proteus).

How pus forms:— When these bacteria infect the tissues, they start an inflammatory process as a result of which, there is always a progressive emigration of leucocytes, followed by the death and liquefaction of many of them, as well as of some of the tissue cells. The result is the production of a creamy opaque fluid which is commonly called *pus*. The dead and the degenerated leucocytes are called *pus cells*. Thus the microscopic appearance of *pus* is a suspension of dead leucocytes in a thick fluid.

Examination of a specimen of pus

1. **Direct smear examination:**—Take a loopful of the material and make some uniform smears. When dry, fix them on the flame and stain as follows:—

(a) Stain with ordinary *carbol-methylene blue* for 2 minutes. Wash under the tap water and dry. Examine under the oil immersion lens. The nuclei of the pus cells and the bacteria will be stained blue.

(b) Take another smear and stain by *Gram's method*. The nuclei of the *pus cells are always stained red* and the bacteria will be either Gram-positive or Gram-negative.

2. **Cultural examination:**—Always verify the result of the direct smear examination by cultural methods. Inoculate into nutrient broth, agar and blood agar; incubate for 24 hours and examine as usual.

Note:— It is always advisable to make [illegible] direct smear examination of a sample of pus before a cultural examina[illegible] undertaken. This will considerably help to decide as to the method [illegible]ation and the nature of the culture media to be used.

ACTINOMYCES OR RAY FUNGUS

These organisms belong to the class of higher bacteria (Chlamydobacteria) and form a connecting link between the bacteria on the one hand and the fungi on the other. They are usually branched, Gram-positive, mycelial filaments showing a star-shaped radial arrangement of the hyphæ, for which the name ray fungus has been given to them (*actin*, a ray; *myces*, a fungus). When introduced into the tissues, they produce a lesion of the character of an infective granuloma, often called *mycetoma*, which literally means a fungous tumour. During the infective process in the animal tissues, they give rise to variously shaped bodies, called grains or granules which are found embedded in the granulation tissue and often escape free in the discharges from the sinuses in the diseased area. These granules have often been compared to poppy seeds, sago grains, sulphur granules or fish roe bodies by different observers.

Distribution:—The organisms of this group are widely distributed in nature, leading a saprophytic existence in the soil and also, as a common parasite of the vegetable world, being present particularly on cereals and grasses. The pathogenic varieties may be found in the mouth and intestines of man and animals, particularly associated with the teeth and tonsils.

Varieties:—

NON-PATHOGENIC—*Actinomyces graminis* (Bostroem): A saprophyte, living on grains and grasses.

PATHOGENIC—*Actinomyces bovis (anaerobic).*

Actinomyces maduræ (aerobic).

ACTINOMYCES BOVIS

HISTORICAL:—*Bollinger*, in 1877, first observed these organisms in the granules secured from the pus discharged from the "lumpy jaw" in cattle. *Israel*, in 1878, first discovered the organisms in human beings in the pus from a case diagnosed as empyema thoracis. He also gave a scientific description of the nature of the lesion and the character of the organisms. *Wolff and Israel*, in 1891, were the first to cultivate the "fungi" successfully in artificial media under anaerobic conditions.

HABITAT:—The organisms are frequently found in the human mouth, specially in the contents of carious tooth cavities and of tonsillar crypts. They are sometimes incorporated in the tartar of teeth which, on being detached, may accidentally lodge in different parts of the body and may thus set up pathogenic lesions.

MORPHOLOGY:—

In tissues:—This is best studied by securing a small "granule" from a discharging sinus, washing it several times in normal saline, preferably made alkaline with 0·5 per cent. caustic potash. The washed "granule" is now mounted in 50 per cent. glycerin on a glass slide with a cover glass, crushing it with a gentle pressure on the latter. When such a preparation is examined under the *low power of a microscope,* two distinct zones are observed. There is a central portion, light-yellow in colour, surrounded by a peripheral zone which is somewhat thick, dark and looks radially striated. When examined under the *high power,* the central portion is found to consist of a tangled mass of mycelial threads from which filaments are found arranged radially, giving rise to the characteristic astral or ray arrangement of the organisms. Towards the extreme periphery, the filaments show terminal swellings, assuming a club-like appearance (Fig. 108). It is due to these latter structures that the characteristic astral arrangement is observed. It should be remembered that these club-shaped bodies are not spores. They are usually well-marked in very chronic lesions and particularly in the ox.

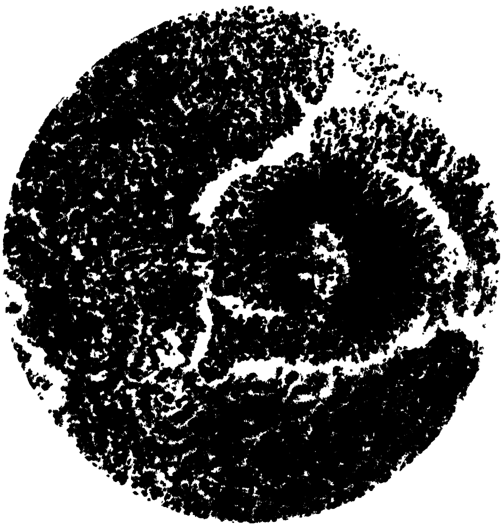


Fig. 108.

A 'granule' under high power, showing the characteristic astral arrangement and terminal clubs of *Actinomyces bovis.* The organism is embedded in a mass of inflammatory cells.

In culture:—The organisms consist of branching mycelial threads, composed of slender, non-septate hyphæ, of varying sizes, from 0·3 μ to 0·1 μ in diameter. The filaments exhibit true branching (Fig. 109a). Sometimes, beaded appearance may be seen, particularly if the conditions for growth are unfavourable (Fig. 109b). In artificial media, it is not uncommon to find the growth more bacillary than mycelial. It is for this reason, that the organisms are often considered to be closely related to the *Mycobacteria.* Clubs are not usually encountered in artificial media but they may appear when they are made to grow in media containing animal fluids.

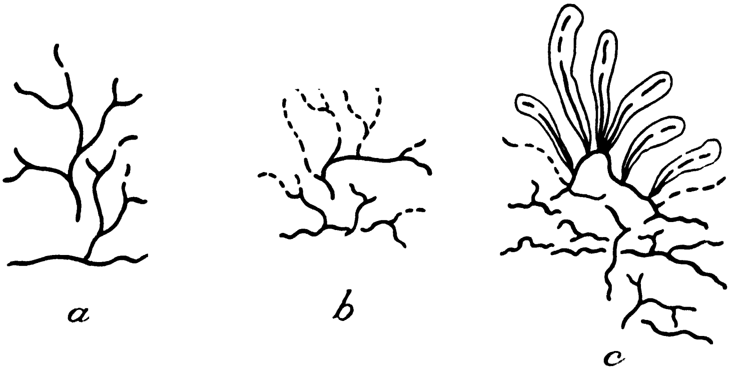


Fig. 109.

Morphological character of *Actinomyces bovis*.

a. filaments exhibiting true branching, b. segmented or beaded appearance of the mycelial threads, resembling bacillary forms; c, clubs inside which the mycelial threads are found to penetrate.

STAINING REACTIONS:—Ordinary aniline dyes do not stain the organisms satisfactorily. They are, however, very well stained by the Gram's method, the *mycelial threads being Gram-positive*, whereas the terminal *clubs, Gram-negative*. They are neither acid-fast nor alcohol-fast.

CULTURAL CHARACTERS: The organisms are *obligatory anaerobes* and grow best at the body temperature. They can be cultivated in all common laboratory media, preferably those containing glycerin and sugar (maltose). There is no growth in MacConkey's medium as the presence of bile salts inhibits the growth of the organism. For the details of anaerobic methods, *vide chapter on anaerobic bacteria.*

Character of growth:

IN FLUID MEDIA:—When the "grains" are put in glucose broth, they are found to increase in size gradually and in the course of 5 to 8 days, appear as tiny compact "mulberry" like granules which may adhere or remain separate but do not disintegrate on shaking. The broth does not become turbid.

ON SOLID MEDIA (Fig. 110):—The organisms grow slowly for 5 to 8 days after which, they appear as small, raised, waxy white colonies of various patterns. When examined under a magnifying lens, these raised colonies are found to have a central elevated portion with depressions and fringed margins at the bases (rosette-shaped or cauliflower-like growth). There is no tendency to spread on the surface but the colonies tend to penetrate deep into the substance of the medium. There is no marked pigmentary change.

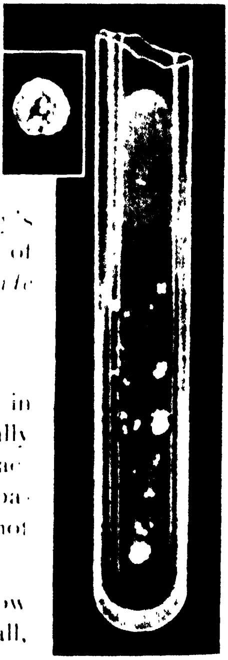


Fig. 110.

Colonies of *Actinomyces bovis* on glucose agar, cultivated anaerobically. The rosette-like appearance of the growth is shown in the inset. (By kind permission of the *Indian Medical Gazette*).

BIOCHEMICAL REACTIONS:—They ferment the common sugars, with the production of acid but no gas. It takes about 3 weeks' anaerobic cultivation to study this reaction. They do not produce any change in litmus milk.

VIABILITY:—Outside the animal body, the organisms cannot exist for a long period. Heating to 60°C. always kills them in 10 minutes. In artificial cultures, they live for 10 to 14 days; in Dorset's egg medium, however, their life is prolonged for 3 to 4 weeks, specially when kept in the cold.

TOXIN PRODUCTION:—Nothing is known about the nature of the toxin.

ANIMAL INOCULATION TESTS:—When the organisms are introduced into the peritoneal cavity of guinea-pigs and rabbits, they produce nodular lesions containing the typical "granules." The lesions are, however, never extensive and the animals do not die.

PATHOGENICITY

Mode of infection:—The exact nature of this process is not known. Though the infectious nature of the disease is not yet definitely proved, yet there is every possibility of the infection being transmitted from one person to another; but this is very difficult to establish.

Source of the organism:—

(a) *Exogenous hypothesis of Bostroem:*—It has been suggested by him that the organism gains entrance into the body through cereals and grasses. But as the causative agent of the actinomycotic lesions in the human or animal body does not exist as a parasite of the vegetable world, this view has

(b) *Endogenous hypothesis:*—The organism normally inhabits the human mouth. It should be noted that the organism helps in the formation of tartar deposit in the teeth in which it can continue its anaerobic existence. Detachment of such tartar and its lodgment in the tissues near about the mouth, alimentary canal and respiratory tracts, can produce actinomycotic lesions. There is no doubt that trauma plays a great part in the development of such lesions.

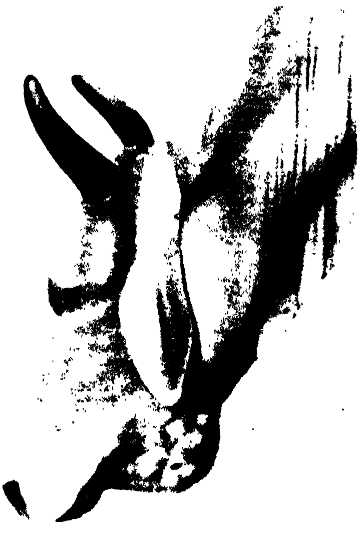


Fig. 111.
Actinomycotic lesion in cattle, showing the 'lumpy jaw.'

Pathogenic lesions:—*IN CATTLE,* the infection is confined to the mouth and jaw, producing a massive granulation tissue. This gives the name " lumpy jaw " or " wooden tongue," according to the parts affected (Fig. 111). The lesions which remain purely local, are mainly productive in nature. There may, however, be suppuration and sinus formation, with discharge of pus, containing the characteristic grains.

IN HUMAN BEINGS, the infection is most commonly limited to the cervico-facial area but may also be found in connection with the respiratory and digestive tracts. Occasionally, the organisms may gain entrance through the abraded skin, producing a very chronic local lesion. Infection by the female genital tracts (ovaries and Fallopian tubes), although recorded, is exceptionally rare. The pathogenic lesions are characterised by *swelling, suppuration and sinus formation*. The discharge contains granules which have been variously compared to fish-roe bodies, sulphur granules, poppy seeds or sago grains. The areas of suppuration are always surrounded by a framework of fibrous tissue and this gives the lesions a characteristic *honey-combed appearance*.

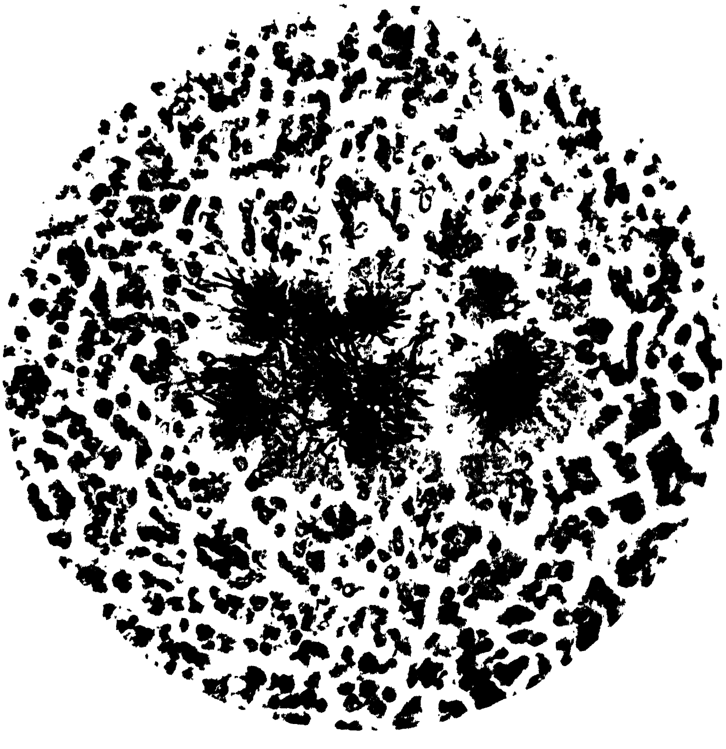


Fig. 112.

Photomicrograph of an actinomycotic lesion in human tissues (kidney), showing the characteristic arrangement of the organism and inflammatory exudates. The clubs are not well marked. Stained by modified Gram-Weigert method.

Under the microscope, three zones may be recognised. In the centre, there is a tangled mass of mycelial threads, surrounded by a ring of regularly arranged filaments, radiating from the common centre. Surrounding this again, there is another ring consisting of club-shaped bodies inside which the mycelial filaments are found to penetrate (*vide* Fig. 109c). All these structures constitute the "granules" which are embedded in a mass of inflammatory exudate, consisting of poly and mono, nuclear, as well as, wandering leucocytes (Fig. 112). Outside this leucocytic zone, there is a framework of connective tissue.

Modes of spread:—This occurs by a process of continuity and contiguity, so that all tissues and organs may be alike invaded. In its progress, the disease may disregard anatomical boundaries and invade one tissue after the other. The lesion breaks down in one direction and heals in another, leaving behind dense cicatricial bands which contract and produce much deformity. The organisms do not spread by the lymphatics and hence, the lymph nodes are usually not enlarged. Metastasis through the vascular system may occur, specially in the late stage of the disease, but it is very rare. In such circumstances, the organisms may enter the blood stream directly or the filaments may be carried away by the mononuclear leucocytes.

Distribution of lesions in human beings:—As already mentioned, actinomycotic lesions are primarily local infections which tend to become generalised. Thus, the various lesions may be divided into two groups—(i) *primary* and (ii) *metastatic or generalised*.

Primary lesions:—These may be found in the following areas and are mentioned in the order of their frequency.

1. *CERVICO-FACIAL AREA*:—The jaw and the adjacent structures are most commonly involved and a swelling appears in the angle or ramus

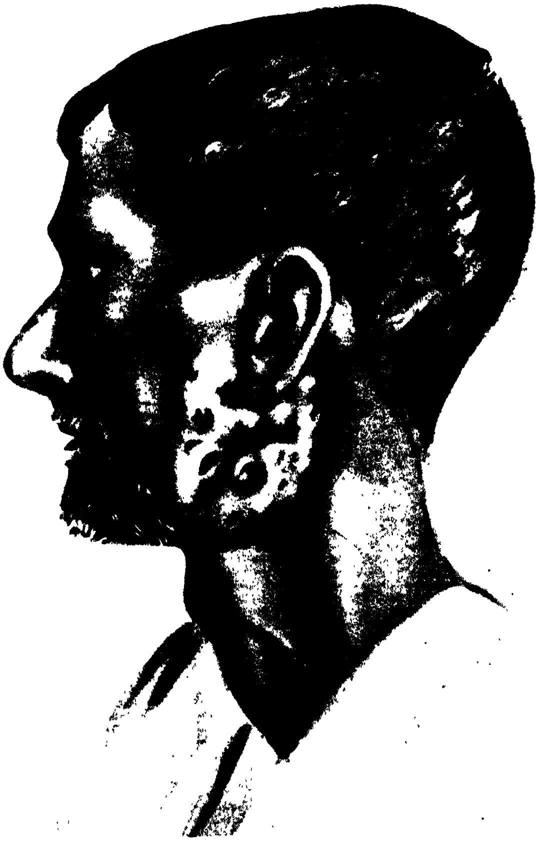


Fig. 113.

Actinomycosis of jaw, showing the typical lesion.

of the mandible, resembling a sarcoma. The presence of a swelling, suppuration and sinuses and the discharge of the characteristic granules will reveal the nature of the lesion (Fig. 113). The final diagnosis is, however, settled by a microscopic examination of the granules.

2. *RIGHT ILIAC FOSSA*:—The favourite site of infection is the cœcum and the appendix. The disease may manifest itself either by an attack of appendicitis or by the formation of a lump in this region. The possibility of the existence of such a lesion can only be surmised, if suspicion is aroused.

3. *THORAX*:—The lungs and the pleuræ may be involved primarily. The signs and symptoms of pulmonary actinomycosis resemble those of pulmonary tuberculosis. The diagnosis can only be settled by a careful examination of the sputum when the actiomycotic threads may be detected.

4. *SKIN* of other parts of the body—This is very rare.

Fig. 114.

Actinomycosis of the female breast.

Note the presence of multiple sinuses and puckered appearance. There is however no retraction of the nipple.

One of us has seen a very interesting case of an actinomycotic lesion of the *female breast*. Clinically, it resembled a scirrhous carcinoma but the presence of multiple sinuses and discharge of the characteristic granules led to the suspicion, the diagnosis being finally confirmed by an histological examination. After the diagnosis, the breast was removed by surgical measures and the appearance of such a breast is shown in Fig. 114.

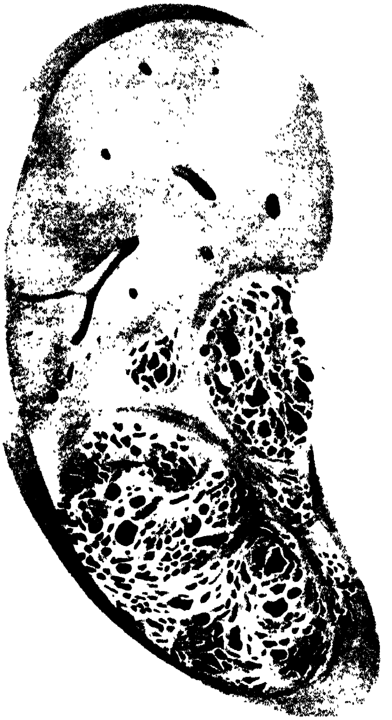


Fig. 115.
Gross appearance of actinomycosis of the liver.

Metastatic or generalised lesions: --The following organs, mentioned in order of their frequency, may be involved: (i) liver, (ii) kidneys, (iii) suprarenals and (iv) brain. The characteristic *honeycombed appearance* of actinomycotic lesions is shown in Figures 115 and 116.

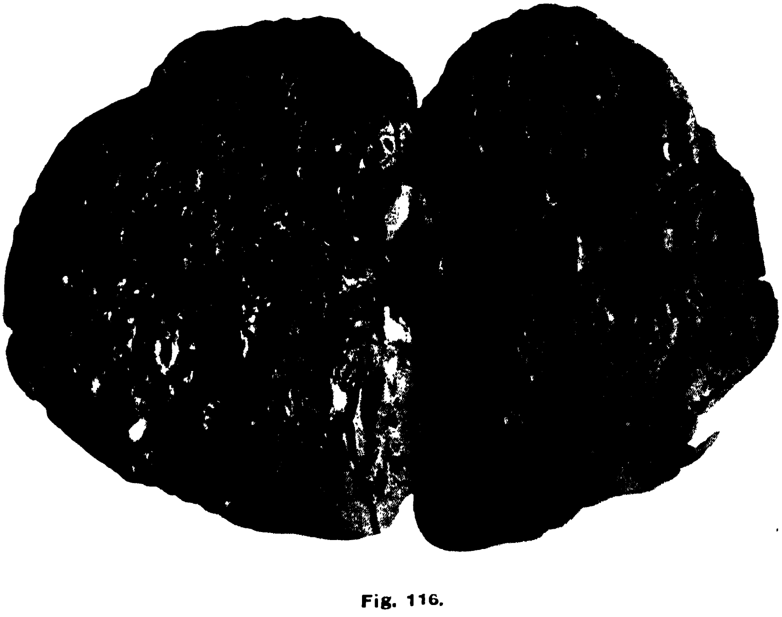


Fig. 116.
Gross appearance of actino the kidney.

THERAPEUTIC CONSIDERATION

Vaccine therapy, preferably with an autogenous one, may be attempted but the results of such treatment are not very promising. Radical surgical measures when practicable should be resorted to. The only drug which is of any value is *potassium iodide*, given in gradually increasing doses, up to the limit of the patient's tolerance.

LABORATORY DIAGNOSIS

Whenever a suspicion of an actinomycotic infection arises, the examination of the pus or any other material should be undertaken with great care. When a physician or a surgeon sends any suspected sample of pus for examination to a laboratory, he should always attach a short record of the case and a description of the lesion from which it is collected. In this way, the attention of the bacteriologist is drawn to the possibility of the infection. During examination, the pathologist must remember that it is the selection of the proper material, viz., the one containing the granules, that ensures success. A casual examination of a random sample of pus is useless. Another mistake which is likely to occur is the omission to examine a film preparation of a sample of pus, when the latter is sent for cultural examination. And the last, but not the least, important cause for a negative result is to forget to culture the material under anaerobic condition.

The organisms are always confined to the "granules" and it is these which should be subjected to a detailed examination. The granules are often found out by spreading the pus in a thin layer over the sides of a test tube or on a Petri dish. Another very practical way to separate the granules from the pus is to shake a portion of the latter vigorously in normal saline with 0·5 per cent. caustic potash in a test tube. In this way, the pus is dissolved in the saline, leaving only the granules which settle at the bottom of the test tube and which may then be easily collected with a pipette.

Methods of Examination: -These comprise a microscopic and a cultural examination.

Microscopic examination:—When an isolated granule is mounted in 50 per cent. glycerin on a glass slide and gently pressed with a cover slip so as to partially crush it, the characteristic structures of the "ray fungus" will be observed. A smear preparation from a crushed granule may be stained very satisfactorily by Gram's method, using dilute carbol-fuchsin as the counter-stain. The mycelial filaments will be found to be Gram-positive while the "clubs" if present, will appear Gram-negative.

Cultural examination:—Some of the granules, after being thoroughly washed in alkaline normal saline, are transferred aseptically into broth, glucose or maltose agar and glycerin agar. The culture tubes are incubated anaerobically at body temperature, when the characteristic colonies may be found to develop in 5 to 8 days.

ACTINOMYCES MADURÆ

The organism was first isolated by *Vincent* in 1894. It produces a chronic productive inflammation which particularly affects the foot. The disease is very prevalent in warmer countries and in certain parts of India, such as Madura, a district in the Madras Presidency, where the disease is found to have the greatest incidence. Owing to all these characteristics, the disease is called "Madura foot." The separate clinical entity of this disease was first proved by *Vandyke Carter* (1860) in Bombay.

In ***morphology*** and in their ***staining*** characters, the organisms very closely resemble Actinomyces bovis.

Culturally, the organisms grow in all media but only under aerobic condition and thus differ from Actinomyces bovis which grows only under anaerobic condition. The growth may take place at 20°C.

IN LIQUID MEDIA: The organism grows in the course of 5 to 8 days and appears as so many *tiny puff balls* which fall at the bottom and remain separate (Fig. 117). The upper part of the broth remains clear.

IN SOLID MEDIA: The colonies heap up in 5 to 8 days' time in a peculiar fashion resembling so many *earthworm casts on the surface of the ground* (Fig. 118). The colonies often show a pink colour. They may be black or red, according to the respective varieties.

Biochemical reactions: It does not ferment any sugar. In litmus milk, it produces an alkaline reaction.

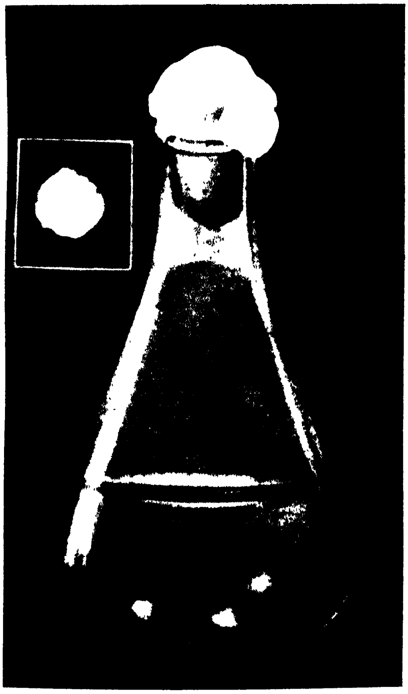


Fig. 117.

Colonies of *Actinomyces maduræ* in glucose broth cultivated aerobically. the [illegible] of the growth shown in the inset. (By kind permission of the Indian Medical Gazette).

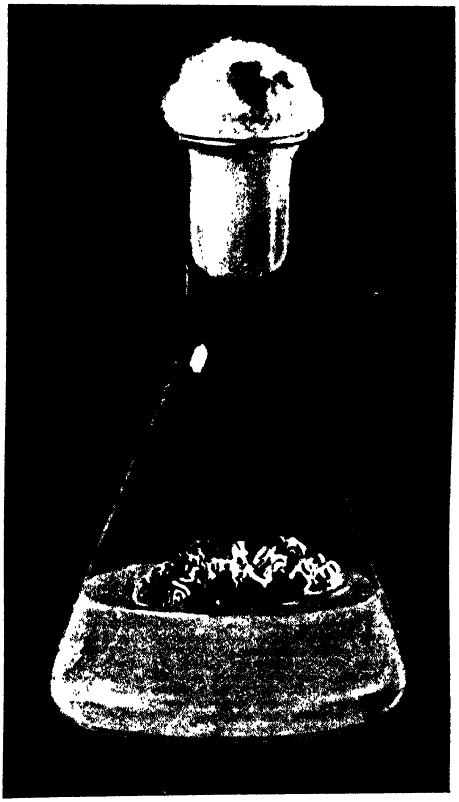


Fig. 118.

Colonies of *Actinomyces maduræ* in maltose agar cultivated aerobically. The earthworm-cast appearance of the growth is very beautifully shown. The dark areas represent the pigmented portions of the growth.

Chromogenic property is well marked and, according to the nature of the pigment formation, several varieties have been recognised, such as (1) white or pale, (2) yellow, (3) black and (4) red.

Animal inoculation:—This is negative as the organisms are, as a rule, even less pathogenic than Actinomyces bovis.

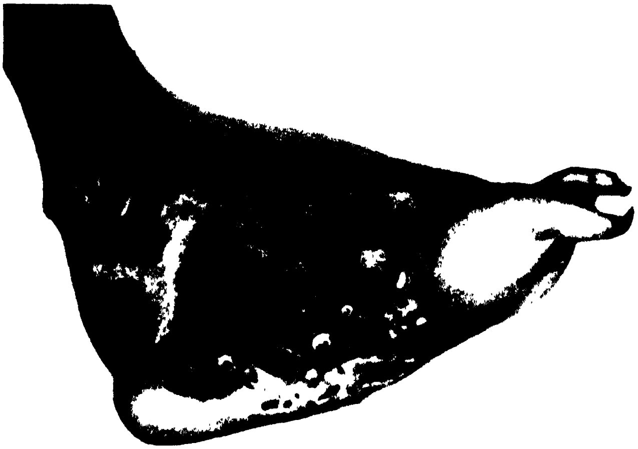


Fig. 119.
Madura foot (Mycetoma foot)

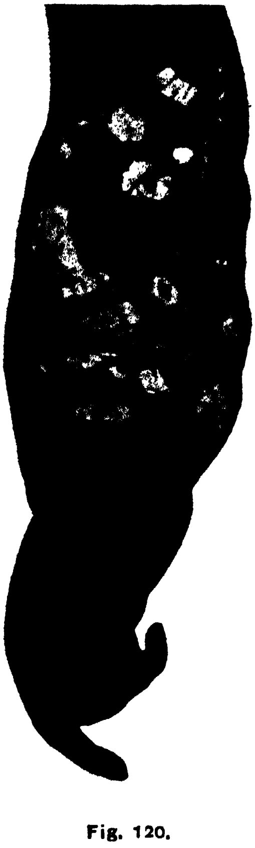


Fig. 120.
Mycetoma forearm.

Pathogenicity:—The disease is mainly confined to the foot (Fig. 119), less so in the hand (Fig. 120) and rarely in other parts of the body. The characters of the lesions are exactly the same as those of Actinomyces bovis. There is swelling, suppuration and sinus formation with the discharge of the characteristic "granules."

In *MADURA FOOT*, the lesion first commences in the sole of the foot and gradually extends till the whole of it is involved. It is riddled with many long-standing sinuses which discharge pus and the "granules." In a well-established infection, the foot is greatly deformed, with its anatomical land marks distorted. It becomes thickened, considerably swollen and its length diminishes. It becomes, more or less, an ovoid mass, with the toes separated and often directed upwards and the sole becoming convex. The lesions are much more extensive and destructive, associated with the production of much connective tissue. Metastasis never takes place and lesions in the internal organs are never found. The disease can be cured completely by surgical removal. Another peculiarity is that potassium iodide which produces marked benefit in infection with Actinomyces bovis is without any effect in madura disease.

MODE OF INFECTION:—This usually occurs only in rural areas where most of the people do not use shoes. Infection takes place through abrasions in the sole of the foot.

For methods of diagnosis see under A. bovis (p. 208).

The difference between *Actinomyces bovis* and *Actinomyces maduræ* is summed up in the following table:—

	Actinomyces bovis.	**Actinomyces maduræ.**
Conditions for growth.	Anaerobic. No growth at 20°C.	Aerobic. Growth at 20°C.
Characters of growth:		
In fluid media.	Mulberry like mass.	Puff-balls.
In solid media.	Cauliflower like growth; no pigmentation.	Growth like the appearance of earthworm casts and is generally of a pink colour.
Chromogenic property.	Nil	Well marked.
Fermentation reaction.	Ferments many sugars without any gas formation. No change in litmus milk.	Does not ferment any sugar. Litmus milk turns alkaline.
	Intraperitoneal inoculation into a guinea-pig forms localised nodules.	Non-pathogenic.
Pathogenicity:— Distribution.	Mostly limited to the cervico-facial area, but may involve other parts of the body.	Strictly limited to the foot, less so in the hand.
Metastatic lesion.	Present.	Absent.
Effect of potassium iodide.	Beneficial.	No effect.

CHAPTER XVIII

STAPHYLOCOCCI

These are cocci arranged in groups resembling bunches of grapes (*Staphylos*, a bunch of grapes; *coccus*, a seed). This arrangement is due to their mode of division. Here several successive divisions take place in many different planes without following any ordered sequence (*vide* page 12).

HISTORICAL:—*A. Ogston* (1881), a surgeon of Aberdeen, incriminated cocci as the cause of suppuration and succeeded in cultivating the organisms. By experimental inoculation with these cultures and pus, containing cocci, he was able to produce pyogenic lesions in animals. Later (1883), he was able to distinguish two kinds of cocci, (i) cocci in groups and (ii) cocci in chains. The former he named Staphylococci and for the latter, he retained the name of Streptococci, as suggested by Billroth. *Julius Rosenbach* (1884) obtained two types of Staphylococci from abscesses Staphylococcus pyogenes aureus and albus. *Passet* (1885) isolated a third type, Staphylococcus pyogenes citreus. It should be noted that it is with the white colonies of Staphylococci that the *Bacteriophage phenomenon* was first observed by Twort in 1915.

HABITAT:—In the human body, they are found normally in the following situations: —(i) horny layer of the skin and cutaneous glands, (ii) mouth and throat, (iii) anterior nares, (iv) external auditory meatus and (v) anterior part of the urethra (preputial sac).

MORPHOLOGY:—These are cocci which grow in irregular clusters, like bunches of grapes, and are non-motile and non-sporing. When examining a smear of pus, in addition to cocci in irregular masses, single cocci and cocci in pairs may also be observed. Many of them may be seen to be intracellular, being phagocytosed by the polynuclear leucocytes (Fig. 121).

STAINING REACTIONS:—It stains readily with all aniline dyes, such as methylene blue, methyl-violet, carbol-fuchsin, etc. When stained by Gram's method, it retains the violet colour after decolorisation, i.e., Gram-positive. It is non-acid-fast.

CULTURAL CHARACTERS:—It grows readily in ordinary laboratory media, such as nutrient agar and broth. It is an aerobe and also a facultative anaerobe.

On ***agar,*** it produces thick opaque, discrete colonies which are circular discs, being relatively large (Fig. 122). The colour of the colonies will vary according to the variety. If massive inoculation is made, the colonies overlap, resulting in the formation of a thick film.

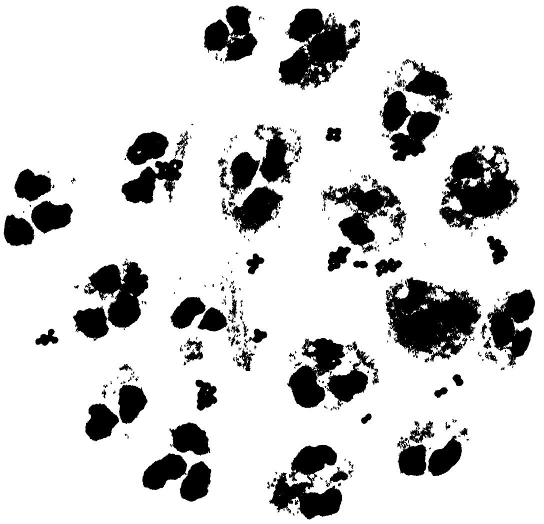


Fig. 121.
Morphology of *Staphylococci*, as seen in a smear

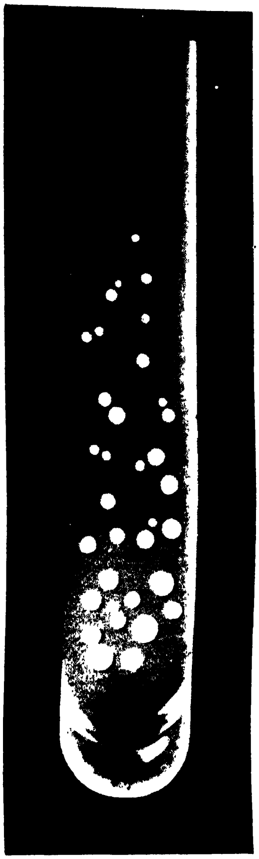


Fig. 122.
Colonies of *Staphylococci* on nutrient agar.

In ***broth***, it produces a uniform turbidity with a deposit at the bottom.

On ***blood agar***, it produces hæmolysis and hæmodigestion

BIOCHEMICAL REACTIONS:—1. It lique-
rium.

2. It produces a pigment (belonging to lipochromes) which does not diffuse into the surrounding medium but colours the colonies only. In this way, according to the nature of the pigment produced, three varieties of the organism were recognised. Those producing yellow colonies were called Staphylococcus aureus, those producing white colonies, Staphylococcus albus and those producing lemon yellow colonies, Staphylococcus citreus.

3. It produces acid but no gas in glucose, maltose, mannite and other sugars. Litmus milk is clotted with the production of acid.

4. The reducing action of staphylococcus is shown by the conversion of nitrate into nitrites and decolorisation of dyes, such as litmus and methylene blue.

VIRULENCE:—The great majority of the strains isolated from suppurative lesions are very virulent and pathogenic for animals. This virulence is fairly well preserved even after numerous subcultures. Passage through animals increases their virulence.

RESISTANCE:—Staphylococci are fairly resistant and stand considerable drying. They have been grown from dried [illegible] after 2 to 3 months. They withstand a temperature of 60°C. for half an hour. Perchloride of mercury is a poor disinfectant for Staphylococci, as a one per cent. solution requires 10 minutes' exposure to completely kill them. Some aniline dyes exert a strongly germicidal effect on them. The most important of these are malachite green (1 in 10,000) and gentian violet (as low as 1 in 2,000,000). It is for this reason that the latter is extensively used in the treatment of superficial pyogenic lesions of the body.

TOXIN PRODUCTION:—Two kinds of toxins are produced by the pathogenic variety of Staphylococci; one is *hæmolysin*, which is capable of dissolving red blood cells and the other is *leucocidin*, a substance capable of destroying white blood cells. Both of them are obtained from the culture filtrate of the organism.

Recently an *exotoxin* has been obtained by Parish and Clark (1932) by growing the organism in special media in an atmosphere containing 25 per cent. CO_2 (*Journal of Pathology and Bacteriology, Feb. 1932*).

SEROLOGICAL PROPERTIES:—When injected into an animal, the following antibacterial substances can be demonstrated in its blood serum, such as *agglutinins, precipitins, immune opsonins (bacteriotropins) and complement-fixing bodies.* There is no definite serological reaction which can be utilised for the diagnosis of Staphylococcal diseases. ***Hine,*** however, utilised agglutination reaction and agglutinin absorption tests in the classification of various strains of staphylococci (*vide infra*). Therapeutic use of such antistaphylococcal sera did not offer any promising result. As already mentioned, attempts have been made to prepare an exotoxin which is utilised in the preparation of antitoxic serum. The latter may, therefore, be used therapeutically and beneficial results have been recorded in some cases.

CLASSIFICATION:—To classify Staphylococci on the basis of pigment production into 3 types, viz., St. aureus, St. albus and St. citreus, is useless, because there are evidences which show that this chromogenic power is not constant and under certain conditions this property is lost. Quite recently, it has been found that serological tests appear to be most satisfactory. *Hine* (1922) found that simple agglutination test is not sufficient to separate these cocci and therefore substituted absorption test. In this way, he has been able to classify Staphylococci into 2 main groups:—

(1) ***St. pyogenes*** (mixed yellow and white colonies) It consists almost entirely of one serological type and generally ferments mannite.

(2) ***St. epidermidis*** (almost all white colonies) This strain does not generally ferment mannite.

It will be noted that the former is more active than the latter in the following respects:—(i) certain biochemical reactions, viz., liquefaction of gelatin and sugar fermentation, (ii) production of toxins (hæmolysin and leucocidin), and (iii) virulence (pathogenic to man and rabbits).

DIFFERENCE BETWEEN THE TWO TYPES OF STAPHYLOCOCCI

Organism.	Broth.	Agar.	Litmus milk.	Mannite	Toxin.	Patho-genicity.
Staphylococcus pyogenes.	Turbid.	Golden yellow or white colonies.	AC	A		
Staphylococcus epidermidis.	Turbid.	White colonies	AC	O	O	Feeble. It is the common cause of stitch abscesses.

A—Acid; AC—Acid and clotting; +—Positive; O—Nil.

ANIMAL INOCULATION:—Of the various laboratory animals, rabbit is the most susceptible. Local abscesses are produced when the organisms obtained from pyogenic lesions are injected subcutaneously and death may follow from septicæmia and pyæmia when they are introduced intravenously.

PATHOGENICITY

Of the various strains, St. pyogenes (aureus) is mostly pathogenic. As the organisms normally inhabit the surface of the skin, it is usual to find the lesions mostly limited to that area. The natural path of infection is through an abrasion in the skin or through the hair follicles and sweat glands, and the usual tendency of such an infection is to localise and form pus in and under the skin. Staphylococcal infection is associated with an emigration of many neutrophilic polymorphonuclear leucocytes which surround the infected area. The leucocidin produced by the organisms destroys many of the emigrated leucocytes, as well as some of the tissue cells, resulting in the formation of a necrosed mass in the centre, surrounded by a thick layer of leucocytic barrier. Ultimately, liquefaction occurs, forming a "core" of pus.

The following is a list of the common ***local lesions*** caused by Staphylococcus pyogenes (aureus).

Common boils or furuncles:—These are deep seated infections of the hair follicles with St. pyogenes aureus (pustular folliculitis). After an induration, the central part softens, leading to the formation of a liquid pus. If incision is made before actual pus has formed, it may lead to disastrous results by opening up the lymphatics and allowing dissemination of the organism. Hence, though these lesions appear to be simple, much judgment is required in these cases as a faulty surgical interference may bring on a fatal termination.

Carbuncle:—This is a group of boils in which the subcutaneous tissues have become involved in the infective and necrotic processes. To start with, it appears as an ordinary boil which spreads rapidly, resulting in a large, red, indurated and painful area with a diameter of many inches. Later on, numerous points of pus appear on the surface of the swelling which bursts, leading to a cribirform condition of the cutis (Fig. 123).

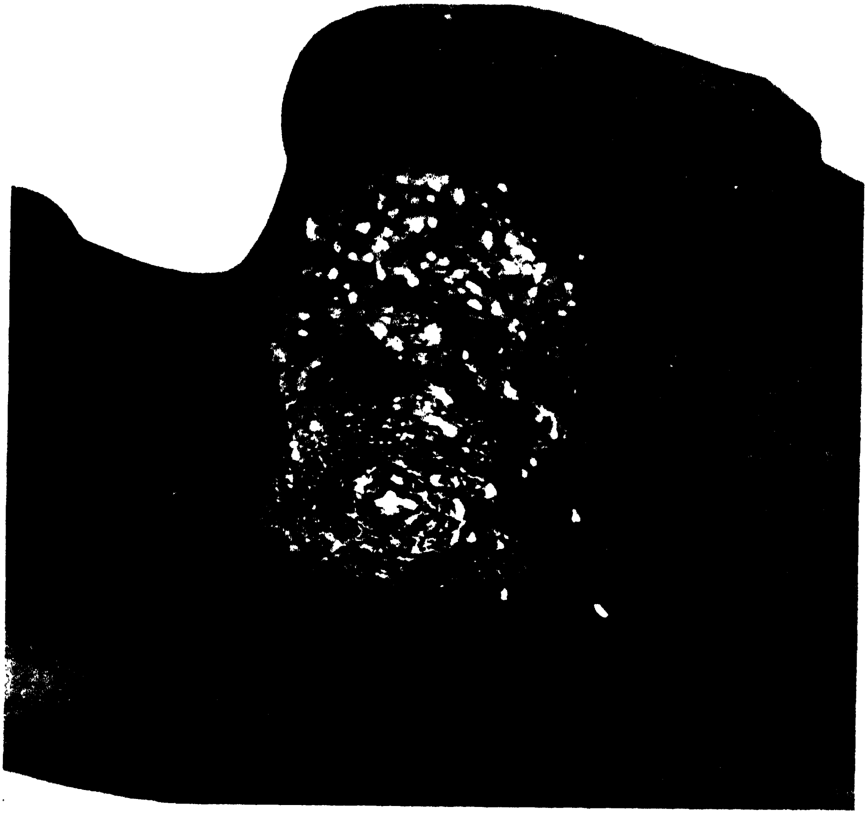


Fig. 123.

A huge carbuncle on the back, showing the cribr form appearance of the lesion.

Whitlow:—This term is applied to an infected wound at the tip of a finger which is extremely painful and usually runs on to suppuration. It may be limited to the finger or may spread along the tendons and their sheaths into the palm of the hand and forearm.

Cellulitis:—This is an inflammation of the subcutaneous tissues and particularly involves those areas which are very loose and traversed freely by lymphatic vessels. The former offer better opportunities for the multiplication of the Staphylococci and the latter aid their rapid spread through tissue spaces. Staphylococcal cellulitis is usually localised and terminates in an abscess formation. It is only under special circumstances that the infection tends to spread. This usually happens when the inflamed tissues are incised before localisation and before actual pus formation has occurred.

General lesions:—Under certain circumstances, the infection tends to generalise and the organisms enter the circulation and may subsequently be deposited in distant parts of the body, producing septicæmia and pyæmia. The conditions which favour this generalisation are:—(i) interference with the defensive mechanisms either through ignorance or through inexperienced surgery and (ii) lowered vitality; the latter being due either to disease, such as diabetes, chronic Bright's disease and long continued illnesses or to overwork, sedentary habits and faulty diet.

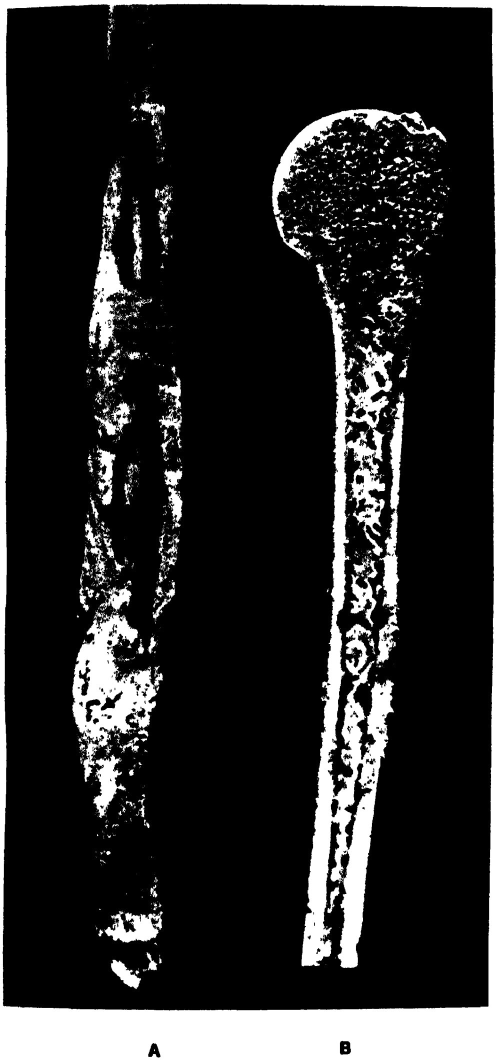


Fig. 124.

Acute and chronic osteomyelitis.

A—Chronic osteomyelitis of the fibula, showing the central necrosed bone (*sequestrum*), surrounded by a new sheath of bone (*involucrum*). The openings in it are called *cloacæ* through which pus was discharged.

B—Acute osteomyelitis of the humerus, showing the conversion of whole of the medullary cavity into a core of pus.

The following are some of the common examples of generalised infection.

(a) **Osteomyelitis**:—Staphylococcus produces an acute inflammatory affection of the bones involving both the medullary cavity and the cancellous tissue. The disease is most commonly met with in unhealthy children with poor resisting power. It frequently follows one of the exanthemata, e.g., measles or scarlet fever. An infected condition of the blood is almost always present before the attack, and foci of local suppuration may be found somewhere in the body. The exciting factor is often a slight trauma, applied in some form, which determines the auto-infection. *Fraser* from a study of 400 cases of osteomyelitis has found that the causative organisms are usually Staphylococci (78 per cent.), but Diplococcus pneumoniæ are found in 14 per cent. and Streptococci in 6 per cent. of his cases. The disease almost always starts in the soft vascular tissue of the metaphysis. From this primary focus, the infection may spread into the periosteum, forming a subperiosteal abscess, as well as into the medullary cavity, destroying all structures in it and converting it [illegible] core of pus (Fig. 124B). The [illegible]ted portion of the bone [illegible] undergo necrosis and remain as a *sequestrum*, surrounded by a mass of newly formed bone (*involucrum*) from the periosteum (Fig. 124A). The infective materials may also be detached and carried into the general circulation as emboli, resulting in pyæmia. The neighbouring joint usually escapes infection, because of the intimate connection between the periosteum of the diaphysis and the epiphyseal cartilage.

(b) **Septicæmia and pyæmia:** Staphylococcal septicæmia is very dangerous and it is particularly so in the tropics. It has been found, on many occasions, that a positive blood culture is invariably associated with a fatal result. Such a septicæmia may arise in connection with a wound infection, a rapidly spreading cellulitis or in a case of a septic uterus during puerperium. Unlike Streptococci, pyæmia, i.e., formation of foci of abscesses, is much more common than general septicæmia.

(c) **Ulcerative endocarditis:**—This is a part of the manifestation of a general septicæmia with localisation of the organisms on the cardiac valves. The character of the lesions is the same as in the case of the Str. hæmolyticus.

(d) **Genito-urinary tract infection:** Kidneys may be involved and cases are on record where an acute nephritis has developed in the course of septicæmia. Usually, the organisms reach the kidneys via the lymphatics, from an infection in the lower urinary tract. This is known as an ascending infection of the urinary tract and it produces pyelonephritis or diffuse suppurative nephritis. Staphylococci are found as secondary invaders in many genito-urinary affections, particularly in gonorrhœa.

(e) **Staphylococcal meningitis:** This may arise in association with general septicæmia. The meninges may also be involved from an extension of the infection from otitis media and osteomyelitis of the skull bones.

Staphylococcal infection of the respiratory tracts:—Staphy do not usually grow on the mucous surfaces and hence staphylococcal infection of the upper respiratory tract is uncommon. Broncho-pneumonia, however, may develop in the course of staphylococcal septicæmia and may be encountered as a secondary complication in influenza.

NOTE:—A chronic infection with long standing suppuration may lead to ***amyloid changes*** in the spleen, kidneys, intestines, liver and lymph glands.

Facial cellulitis (Fig. 125): This is a common example of fatal spreading cellulitis produced by staphylococci and is seen in cases of furuncles of the face and lips. The common facial "erysipelas" condition which we often see in our country is in the majority of instances, not an example of true erysipelas but staphylococcal cellulitis of the face. The condition is very fatal and tragic, and is invariably associated with virulent organisms. The usual history of such a case is the appearance of a small pimple on the lip with redness and swelling, usually of one or two days' duration. When this is pricked or squeezed inadvertently, the organisms, being highly virulent, rapidly invade the blood stream, producing a fulminant septicæmia which invariably results in a fatal issue. A faulty surgical interference at this stage also leads to very serious consequences and the condition, instead of improving, usually grows worse after the incision. The redness and œdema increase very considerably, spreading all over the face, eyelids and even over the scalp. The patient may die of this rapidly spreading cellulitis in a very short time, sometimes as early as 3 to 4 days.

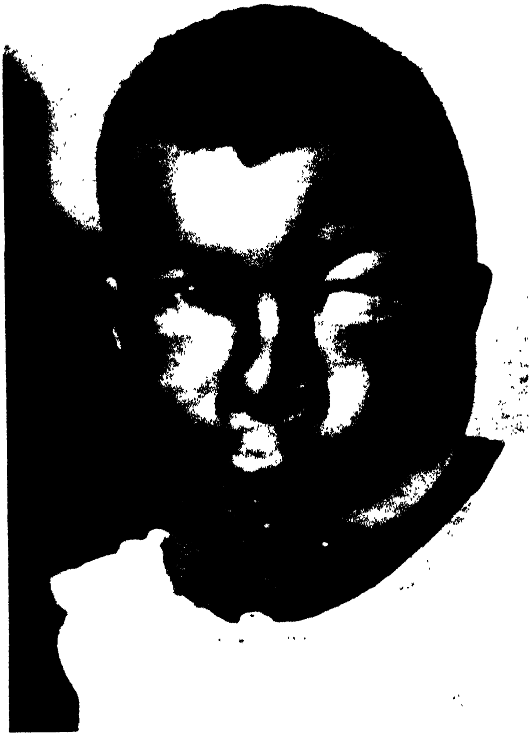


Fig. 125.
Staphylococcal cellulitis of the face. Note the extension of the œdema.

In this connection, it should be remembered that apart from any external interferences mentioned above, the infection may spread, inspite of the most careful and conservative measures. These boils are always attended with an early septic thrombophlebitis of the neighbouring veins. The infected thrombi rapidly spread to the larger veins of the face, particularly the anterior facial. There are certain anatomical peculiarities of the latter, such as the absence of any valve, and its situation amongst the facial muscles which, by constant contractions, displace the clots in the vein and pump them onwards. Moreover, owing to frequent communications of the facial vein with the cavernous sinus (Fig. 126), the infection is easily carried to the interior of the skull, causing either sinus thrombosis (cavernous) or meningitis. Owing to the above reasons, furunculosis of the naso-labial area, as indicated by the shaded area in Fig. 127, is very dangerous.

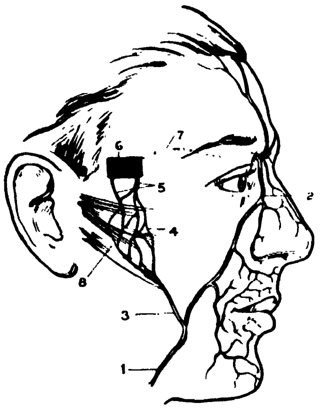


Fig. 126.

Diagram illustrating the communication of the anterior facial vein with the cavernous sinus.
1, anterior facial vein; 2, angular vein; 3, deep facial vein; 4, pterygoid venous plexus; 5, emissary veins; 6, cavernous sinus; 7, superior ophthalmic vein; 8, pterygoidus externus muscle.

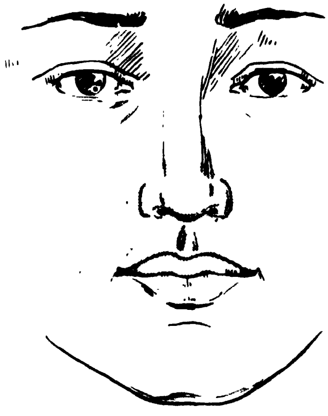


Fig. 127.

he shaded area represents the 'dangerous area' of the face.

Seasonal incidence of Staphylococcal infections in the tropics:—

Virulent staphylococcal infections are very common in the tropics during the summer months and in the rains. This may be attributed particularly to the humidity of the atmosphere causing constant perspiration which cannot evaporate rapidly. This leads to a sodden condition of the skin and removes its power of auto-sterilisation and of acting as a mechanical barrier. Persons with diabetes are peculiarly liable to suffer from various skin troubles during this period, as staphylococci which habitually live upon the skin and form a part of its normal bacterial flora, can easily infect such an altered epithelial surface.

Staphylococci as secondary invaders:—In many superficial cutaneous infections due to other organisms, involving loss of continuity of the epidermis, staphylococcal infection may be superimposed. For example, impetigo contagiosa which is caused by Streptococcus hæmolyticus may be secondarily infected with staphylococci. In respiratory tract infections, particularly in influenza, staphylococi, as secondary invaders, may be responsible for broncho-pneumonia. As secondary invaders, they are also most commonly found in connection with the genito-urinary tract infection. The rôle of staphylococci in gonorrhœal infection is discussed under Neisseria gonorrhœæ.

THERAPEUTIC CONSIDERATION

Serum therapy:—Although antistaphylococcal serum may be produced containing anti-hæmolysins and anti-leucocidins, such a serum does not appear to confer any marked degree of immunity. Recently, an antitoxic serum has been prepared, the use of which is said to produce beneficial results in some cases of staphylococcal infection.

Vaccine therapy:—This, no doubt, increases the opsonic index and produces antibacterial substances in the serum, thus conferring a certain amount of immunity. Such vaccines may be used for curative purposes, preferably in subacute and chronic infections. The usual dose is 20 to 40 millions of the organisms, but in cases of generalised furunculosis, it is better to use smaller doses in the beginning and gradually work it up. It is always advisable to use autogenous vaccines wherever possible.

Staphylococcus anti-virus (p. 119):—An eight to ten days' old broth culture is filtered and heated at 60°C. for 1 hour to reduce its toxicity. This is then used as a compress over the affected part. This produces a local immunity of the area over which it is applied. As a therapeutic agent, it is used in cases of superficial infections of the skin.

Other varieties of Staphylococci

1. *Micrococcus ureæ*—This is a variety of Staphylococcus producing white colonies (St. albus). Its chief feature is that it is capable of utilising nitrogen from the urea. The organism secretes an enzyme which ferments urea with the formation of ammonium carbonate.

2 *Micrococcus lysodeikticus*—This is also a variety of Staphylococcus and may be found as air contamination on culture plates. Its importance lies in the fact that lysozyme which is present in human tears and cartilages is capable of dissolving a thick suspension of this organism in a few seconds (p. 111).

GAFFKYA TETRAGENA (*Micrococcus tetragenus*)

This organism was first recognised by Gaffky (1881). Owing to a certain serological relationship, it is regarded as a variety of Staphylococci. The organism is characterised by its arrangement in tetrads, which depends on the mode of division (p. 12). The apposed surfaces of the cocci are flattened and each group is surrounded by a capsule, when growing in the tissues. It is Gram-positive and stains readily with all the ordinary bacterial stains.

It grows readily on all ordinary media. On agar the growth resembles that of white colonies of Staphylococci but has a viscid character. On blood agar the colonies are surrounded by a greenish zone like Str. viridans. Gelatin is not liquefied.

The white mouse is very susceptible and a general septicæmia results on subcutaneous injection, the capsulated tetrads being found in large numbers in blood throughout the body. In human beings it possesses a feeble pathogenicity and is found normally in the upper respiratory tract. On certain occasions it may be responsible for minor catarrhal inflammations.

SARCINA VENTRICULI

This was first described by Goodsir in 1842. It is found in the contents of the stomach, specially when dilated. Th [illegible] ganism in association with Lactobacillus boas-oppleri (Boas-oppler ba [illegible] s frequently reported in the stomach contents of carcinoma gastri. [illegible] ganism is characterised by an arrangement in cubes or like packets o [illegible] bales. This is due to the division taking place in 3 planes at righ [illegible] to one another. It is Gram-positive.

CHAPTER XIX

STREPTOCOCCI

These are so called because they are arranged in chains, either short or long. Such an appearance is due to their characteristic cell-division. Here, a series of divisions takes place in planes parallel to one another and the daughter cells do not immediately separate but remain attached to one another by their cell-membranes forming a chain (*vide page 12*).

HISTORICAL:—In 1881, *Ogston* first differentiated between the grouped and the chained cocci in human lesions. The name Streptococcus was however given by Billroth (1874) (*Streptos*, chain and *coccus*, a seed). Pure cultures of Streptococci were first obtained from erysipelas by *Fehleisen* (1883). *Marmorek* first produced and studied the properties of antistreptococcal serum.

VARIETIES:—The following are the various strains of Streptococci which are commonly found in human beings.

1. *Streptococcus hæmolyticus.*
2. *Streptococcus viridans (Mouth streptococci).*
3. *Streptococcus pneumoniæ or Streptococcus mucosus of Schottmuller.*
4. *Streptococcus fæcalis or Enterococci of Thiercelin (Bowel streptococci).*

HABITAT:—Many of the species of Streptococci are encountered normally in the mouth, upper respiratory tracts, about the genitalia (lower part of the vagina) and in the intestines. They are not usually found on the normal skin. *Streptococcus viridans* is found as a saprophyte upon the mucous membranes of the mouth, pharynx and the upper respiratory passages of man. Streptococci may be present in the vagina but they are mostly of the non-hæmolytic type. *Streptococcus hæmolyticus* may rarely occur as commensals in the throat and, under certain conditions, may serve as a source of endogenous infections. *Streptococcus fæcalis* is constantly present in the stools and it is one of the most frequent members of the bacterial flora in the lower part of the bowels. *Streptococcus pneumoniæ (mucosus)* may sometimes be encountered normally in the upper respiratory tracts of man.

MORPHOLOGY:—These are cocci usually spherical and are arranged in short or long chains, or sometimes in pairs. They are non-motile and non-sporing. The majority have no capsules, except Streptococcus pneumoniæ.

Size:—Each coccus is about 0·75 to 1 μ in diameter.

Arrangement:—They are arranged in chains, composed generally of ten or more individuals (Fig. 128). These vary in length depending largely on the environment. At one time, these cocci were classified according to the length of their chains, e.g., Str. longus (long chains) and Str. brevis (short chains); Str. hæmolyticus belonging to the long chain variety while Str. viridans to the other. Str. fæcalis (Enterococci) occur as diplococci or in short chains.

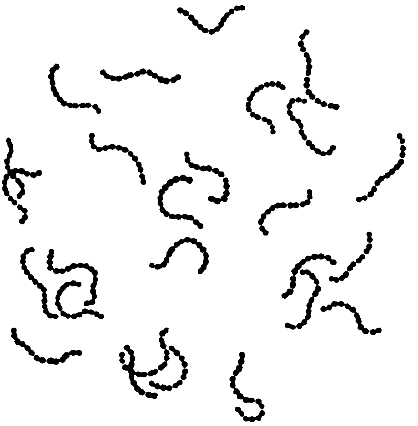


Fig. 128.

Morphology of *Streptococci* haemolytici showing the arrangement of the organism in chains. Smear preparation from a young

Note:—Chain formation is usually well developed in fluid media.

STAINING REACTIONS:—They stain readily with all aniline dyes. With Gram's stain, they retain the colour (Gram-positive).

CULTURAL CHARACTERS:—The organisms are aerobic and facultatively anaerobic. They grow readily in ordinary artificial media, such as agar and broth; but the addition of serum or blood greatly facilitates their growth and an excellent growth may be obtained on serum agar or blood agar. In the latter medium, the hæmolytic activities of the Streptococci are also studied.

Characters of growth:—

On solid media, the colonies are small, delicate, slightly transparent and discrete (Fig. 129). In broth, the growth forms a floccular deposit at the bottom of the tube, leaving the upper part clear. This is very characteristic.

Selective methods for isolation:—These methods are particularly useful in isolating streptococci from a mixed infection. When grown in ordinary culture media they are over-grown by the more vigorous secondary organisms, such as staphylococci. The addition of crystal violet in 1 : 100,000 to 1 : 500,000 to agar or bouillon markedly inhibits the growth of staphylococci but not that of streptococci (Hexthausen's method, 1927).

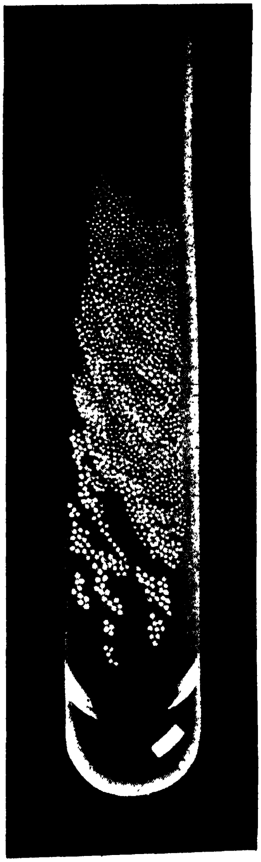


Fig. 129.

Colonies of *Streptococci* on serum agar; pure culture.

Further, it has been observed that partial anaerobic condition is favourable to streptococcal but unfavourable to staphylococcal growth (Sabouraud's method, 1892, 1900).

BIOCHEMICAL REACTIONS:—

(1) ***Proteolytic power:***—They are not actively proteolytic and therefore there is no liquefaction of gelatin and no indol formation.

(2) ***Sugar fermentation:***—Hydrolysis takes place with the production of acid but no gas. This fermentative capacity varies with different species of Streptococci as shown in the table below:—

	Str. hæmolyticus.	Str. viridans.	Str. pneumoniæ.	Str. faecalis.
Lactose				
Salicin				
Mannite				
Raffinose				
Inulin (Polysaccharide) ...				
Aesculin (Glucoside) ...	-	–		+

+ means fermentation with production of acid but no gas.

- means no fermentation.

(3) ***Hæmolytic activity of Streptococci:*** On *blood agar*, some strains (Str. hæmolyticus) have the power to produce a diffusible hæmolysin which gives rise to a clear zone around the colonies. The amount of hæmolysis in blood agar, however, varies according to the strains used.

(i) When the hæmolysis is very marked, producing sharply defined clear colourless zones, it is called β-hæmolysis, as in Streptococcus hæmolyticus (Fig. 130).

(ii) When the hæmolysis is not so marked but is only partial, producing a greenish discoloration around the colonies, it is called α-hæmolysis, as in Streptococcus viridans (Fig. 131) and Streptococcus pneumoniæ.

(iii) When the organism is entirely devoid of hæmolytic property and no green discoloration is produced on a blood medium, it is called γ-hæmolysis, i.e., no hæmolysis, as in Streptococcus fæcalis.

As already mentioned, three factors are concerned in the causation of complete laking of the blood round the colonies of Streptococcus hæmolyticus and they comprise the following:—

(a) Absorption of the blood pigment into the colonies.

(b) Destruction of the blood pigment which is converted into a colourless compound by a filterable hæmolysin.

(c) Migration of the pigment at the periphery of the clear zone, due to a change in the reaction (pH5) in the immediate vicinity of the colonies (Rother).

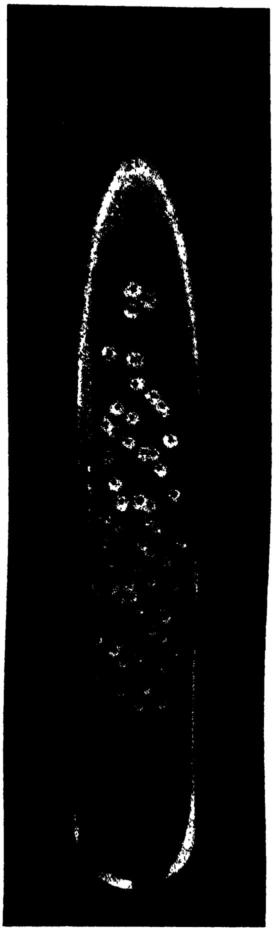


Fig. 130.

Colonies of *Str. hæmolyticus* on blood agar, showing the formation of clear zones (β-hœmolysis) around the colonies.

The green colour noticed in and around the colonies of Str. viridans is due to the formation of methæmoglobin which is an oxidation product of hæmatin. It should be noted that on blood-agar, D. pneumoniæ also forms green colonies without any hæmodigestion. Such a change in the colour is brought about by the action of H_2O_2 (produced by these latter organisms during their growth) which oxidises hæmoglobin to methæmoglobin.

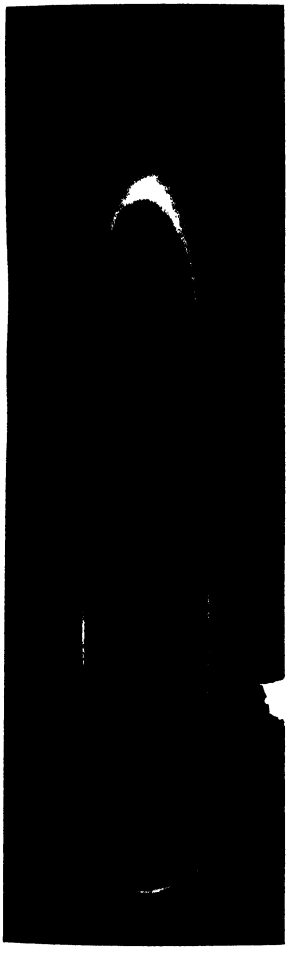


Fig. 131.

Colonies of *Str. viridans* on blood agar. Note the partial hæmolysis (α-hæmolysis), green coloration and the pin-point colonies.

According to the nature of this hæmolytic activity, *Smith and Brown* (1919) classified Streptococci into 3 types:—

1. Type β (*beta*)—Frankly hæmolytic, e.g., Str. hæmolyticus.

2. Type α (*alpha*)—Weakly hæmolytic and methæmoglobin-forming (Green producers); e.g., Str. viridans and Str. pneumoniæ.

3. Type γ (*gamma*)—Neither hæmolytic nor methæmoglobin-forming; e.g., Str. fæcalis.

Lastly, it should be remembered that this power of hæmolysing red blood corpuscles serves, to some extent, as an index of the virulence of the strain. The stronger the hæmolytic activity, the greater is its pathogenic power and hence, Str. hæmolyticus is the most virulent, whereas Str. fæcalis is the least so.

NOTE:—The term hæmolysis is, however, not a very suitable expression to describe the clearing of the pigment. The red blood corpuscles are not only laked by the hæmolysins but the liberated pigment is actually destroyed and converted into a colourless substance. Hence, the term hæmodigestion is more appropriate and should be employed.

VIABILITY:—

(1) *Resistance to heat:*—Many species of Streptococci belonging to the hæmolytic group are very susceptible to heat, while the Str. fæcalis resists heating considerably. Broth cultures of Str. fæcalis survive exposure to 55° to 60°C. for 30 minutes (Dible 1921), while the other varieties of Streptococci are killed in 5 to 10 minutes.

(2) *Other destructive agents:* Resistance of the various species to other unfavourable conditions runs parallel to their heat-resisting property.

(3) *Longevity in culture:* For prolonged maintenance in artificial cultures, Str. hæmolyticus and Str. pneumoniæ require frequent subcultures. Str. fæcalis, however, is easily kept in artificial culture for prolonged periods.

VIRULENCE OF STREPTOCOCCI: This varies with different strains and is usually tested on susceptible animals, such as mice. *Str. hæmolyticus* is highly pathogenic to mice, less to rabbits and still less to guinea-pigs. *Str. viridans* is usually non-pathogenic to laboratory animals. *Str. pneumoniæ* is pathogenic to many laboratory animals and its pathogenicity to mice, rabbits and guinea-pigs is the same as that of Str. hæmolyticus. The virulence of *Str. fæcalis* to experimental animals is uncertain. The consensus of opinion is that it is relatively non-virulent; in fact, most strains can be injected in large doses without any pathogenic effects.

Virulence of a given strain may be attenuated by prolonged cultivation in artificial media, but if it is frequently sub-cultured in a medium containing blood, the attenuation is least marked. By frequent passages through a susceptible animal, the virulence is exalted for that particular animal, although that for other animals may be decreased.

TOXIN PRODUCTION:—The bodies of Streptococci are themselves poisonous and such toxic products (endotoxins) are liberated by the death and disintegration of the organisms. Apart from this, Streptococcus hæmolyticus like staphylococci produces two other soluble products, *hæmolysin* and *leucocidin*, during its growth. Dicks (1924) have obtained a filterable toxic product from scarlatinal strains of Str. hæmolyticus, which produces a specific skin reaction (*erythrogenic toxin*).

SEROLOGICAL REACTION:—Antisera have been prepared against Streptococci by immunising horses. Such sera appear to be anti-bacterial rather than antitoxic and contain agglutinins and bacteriotropins. Lately, an antitoxic serum has been prepared against the hæmolytic group of streptococci, producing scarlet fever. There are no such serological reactions which can be utilised in the diagnosis of streptococcal diseases.

CLASSIFICATION:—This may be done on the basis of the following:—(i) *morphological characters*, (ii) *fermentation reactions*, (iii) *serological reactions* and (iv) *hæmolytic activities*.

(i) *Morphological characters:*—Attempts were made to classify Streptococci on the nature of chain formation (long or short) and the presence or absence of capsules. But such a classification was found to be useless and hence given up.

(ii) *Fermentation reaction:* Many attempts have been made to classify Streptococci by this method. The value of fermentation reactions as a differential criterion cannot be utilised, as it is so variable.

(iii) *Serological reactions:* Serological grouping of Streptococci has been tried extensively, but results are not satisfactory. Many attempts have been made to classify hæmolytic Streptococci, particularly on serological basis, and they have been divided by Gordon into 3 groups.

Str. pyogenes producing typical acute infections, frequently terminating in septicæmia and pyæmia.

Str. erysipelatis producing erysipelas.

Str. scarlatinæ causing scarlet fever.

Recent work on the antigenic structure and agglutination reactions of hæmolytic Streptococci has shown that such a rigid distinction into serological types cannot be permitted. Moreover, observations from clinical and laboratory findings tend to show that any one of the organisms from the above three groups is capable of producing any of the infections typical of hæmolytic Streptococci. Thus the serological methods do not offer any great help in classifying Streptococci.

(iv) *Hæmolytic activities* (changes produced in blood agar):—This method has lately been followed in the classification of Streptococci, as it has been found to be far more constant than the fermentation and serological reactions (*vide page* 225).

The following is a summary of the biological classification of the whole group of Streptococci:—

1. *Markedly hæmolytic (Type β of Smith and Brown), e.g., Streptococcus hæmolyticus.*

2. *Slightly hæmolytic or green producers (Type α of Smith and Brown), e.g., (a) Streptococcus viridans and (b) Streptococcus pneumoniæ.*

3. *Non-hæmolytic—*

   (a) Aerobic streptococci, e.g. Streptococcus fæcalis (Type γ of Smith and Brown).

   (b) Anaerobic streptococci.

Table showing the distinguishing features of individual Streptococci.

	Streptococcus hæmolyticus.	Streptococcus viridans. (Mouth streptococci).	Streptococcus pneumoniæ. (Str. mucosus of Schottmuller).	Streptococcus fæcalis. (Enterococci of Thiercelin).
Morphology :— Arrangement ...	Long chains	Short or long chains	Diplococcal or short chains	Pairs of ovoid cocci or short chains
Capsule formation	Absent	Absent	Markedly present	Absent
Growth on blood agar	Marked hæmolysis (β-hæmolysis)	Slight hæmolysis. Methæmoglobin or green producers (α-hæmolysis)	Slight hæmolysis. Methæmoglobin or green producers (α-hæmolysis)	No hæmolysis. (γ-hæmolysis)
Aesculin test (Splitting of glucoside æsculin) ...	–	–	–	+
Mannite fermentation ...	Chiefly	–	–	+
Inulin fermentation	–	–	+	–
Heat resistance (60°C. for 30 minutes) ...	–	–	–	+
Bile-solubility ...	–	–	+	–
Growth on MacConkey's plate ...	– (No growth)	– (No growth)	– (No growth)	+ (Growth)
Pathogenicity for mice ...	Variable; often +	Low	High	Low

+ indicates positive reaction; – indicates negative reaction.

It may be seen from the above table that Str. pneumoniæ corresponds very closely to type III Diplococcus pneumoniæ (pneumococcus). Bile-solubility, inulin fermentation and high virulence for mice suggest that it is a true D. pneumoniæ. It is a slightly larger coccus than the other more typical D. pneumoniæ and is commonly oval or spherical rather than lance-shaped. There is a considerable tendency to form chains. Capsule formation is well marked. The colonies are larger and moister than those of D. pneumoniæ and have a slimy consistency. This stickiness is explained by the marked development of capsules by these organisms.

ANIMAL INOCULATION:—The effects of inoculation vary to a great extent and depend on the following factors—(i) susceptibility of the animal, (ii) routes of inoculation and (iii) degree of virulence of the strain. Of all animals, mice and rabbits are the most susceptible. The more common methods of inoculation are subcutaneous, intravenous and intraperitoneal. When a sufficiently virulent strain is injected, it gives rise to a general septicæmia, with death occurring within 48 hours. Streptococci are found in heart's blood after death. With less virulent strains, septicæmia is not so marked but there is a considerable tendency to localisation in the tissues.

The fate of the inoculated Streptococci has been carefully studied by *Hopkins and Parker* (1918), using cat as a non-susceptible and rabbit as the susceptible animal. They observed that in both cases, the organisms were rapidly removed from the circulation and were held up in the capillaries of the internal organs and in the case of the non-susceptible animal, readily destroyed in the liver and lungs. But in the case of the rabbit, they multiplied in the muscle capillaries, from which the organisms re-entered the general circulation, causing septicæmia. Thus it will be noted that in a susceptible animal, septicæmia is the result of a more or less continuous discharge of bacteria from a focus of infection which is in close or direct communication with the blood stream.

PATHOGENICITY OF STREPTOCOCCI

There are many records of streptococcal diseases *IN ANIMALS*. Among some of the larger domestic animals, such as *horses*, it produces an acute nasopharyngeal catarrh ("Strangles"). In *cattle*, the organisms are most frequently associated with the inflammation of the udder (mastitis); in such conditions, the milk is often infected, giving rise to epidemics of sore-throat in human beings. Among the *smaller laboratory animals*, the commonest type of disease encountered is a septicæmia of obscure origin, associated with marked splenic enlargement and often with evidence of hæmolysis in the general circulation.

IN HUMAN BEINGS, Streptococci produce various kinds of lesions, the nature of which will vary according to the particular strain.

PATHOGENICITY OF STREPTOCOCCUS HÆMOLYTICUS

Rosenbach (1884) first pointed out that the lesions produced by a hæmolytic strain are usually less localised, tend to spread and are more often fatal than those caused by other pyogenic bacteria. The severity of a streptococcal lesion, however, depends on two factors, viz., (i) the invasiveness of the microbic strain, i.e., their virulence and toxigenic properties and (ii) the local and general resistance of the individual host. The remarkable property of spreading and invasiveness can be explained by the fact that there is hardly any satisfactory mobilisation of the phagocytes in infection with virulent streptococci. Moreover, leucocidin produced by the organisms in the early stages, kills the phagocytes that may happen to aggregate at the site of the infection. Finding no barrier, the micro-organisms invade tissue spaces and lymphatic channels with great rapidity, resulting ultimately in septicæmia. Thus, the exudate from a fatal streptococcal lesion appears to be serous rather than purulent and contains few pus cells. When, however, definite pus has formed, the prognosis is better, as it signifies the mobilisation of leucocytes and the formation of a protective barrier. It may be remembered that a virulent strain of hæmolytic streptococci may gain entrance through a minute skin abrasion, such as a prick or a scratch, and produce a fulminant septicæmia with little or no inflammatory reaction at the site of entry.

As the organisms do not usually inhabit the human body, the source of infection, in most cases, is exogenous, and only in some cases, it may be endogenous. The various lesions produced by Streptococcus hæmolyticus may be classified as follows:—

1. *Infection of the skin, subcutaneous tissues and lymphatics.*
2. *Infection of wounds.*
3. *Infection of the respiratory tracts.*
4. *Infection of the genito-urinary tracts.*
5. *Septicæmia and pyæmia.*

Infection of the skin (superficial cutaneous infection) Streptococcus hæmolyticus is not an inhabitant of the normal skin and hence, the infection, in most cases, is exogenous. The organisms tend to produce a serous exudate into the skin and in cases of superficial infections, effusions take place under its horny layer with the production of vesicles; whereas staphylococcus infections tend to occur in the neighbourhood of the hair follicles and are accompanied by a purulent exudate from the beginning. The commonest of these superficial infections are the following:

Impetigo contagiosa: This is an affection commonly seen in children, on their exposed parts, such as hands and face. The lesions consist of small pustules formed between the horny and Malpighian layers of the skin, which often rupture and, when the exudate dries up, leads to the formation of yellowish crusts. These are numerous, often confluent and asymmetrical in distribution and are obviously spread by local inoculation. As a result of scratching, the organisms are often carried to distant parts of the body and thus new crops of lesions appear on the hands, arms, legs and trunk. Doubt was raised as to the etiological relationship of the streptococci to this disease, as ordinary cultural methods were often unsatisfactory for their isolation, the organisms being overgrown by staphylococci. Recently, the use of selective cultural methods has greatly facilitated their isolation and has confirmed their causal relationship.

Paronychia: This is an infection of the semilunar fold at the base of the nail.

Erysipelas: This is an infection of the lymphatics and tissue spaces of the cutis in which the organisms multiply. Vivid rosy red colour with raised margins appears at the site of infection. In coloured patients, however, the rosy red colour is replaced by a reddish brown tint. Swelling is not marked except in lax areolar tissues. Vesicles may form superficially and are generally found near the edges. Suppuration is uncommon. The lesion continues to advance more or less rapidly and generally spreads centrifugally, the central parts tending to fade as the affected area widens. The organisms are usually found crowded in the lymphatics at the spreading margin. The cellular infiltrations are chiefly composed of mononuclear cells which abound in large numbers at the margin. The neighbouring lymph glands are usually involved and there may be a certain amount of lymphangitis, characterised by wavy red lines. The constitutional symptoms are due to the absorption of toxin and, in fatal cases, a general septicæmia supervenes. This condition commonly affects the face and head but may also be seen in other parts of the body. Cases of facial erysipelas often result from the extension of infection from the nose and the nasal sinuses. Infection is believed to take place through a breach of the integument, though in some of the so called idiopathic forms, no such external abrasions can be observed. Originally, it was supposed to be due to a special strain of Streptococci, Str. erysipelatis, which now appears to be identical with Str. pyogenes (hæmolyticus).

Cellulitis: See under Staphylococcus.

Ludwig's angina: This is a condition of cellulitis of the submaxillary region of the neck, resulting from an extension of infection in the mouth or throat.

Lymphangitis: This is an inflammation of the peripheral lymphatic vessels which stand out as wavy red lines in the skin. The larger lymphatic vessels in the deeper parts of the skin and subcutaneous tissues are involved and thus the condition differs from erysipelas where the small lymphatic vessels in the corium are the seats of inflammation. In this connection it should be noted that such streptococcal infection is often associated in the tropics with filarial lymphangitis.

Wound infections: —This is of frequent occurrence in the post mortem rooms and operation theatres and is therefore familiar to pathologists and surgeons. The injuries received may not be necessarily severe, as a small unobserved cut or a minute prick through the rubber gloves, received during the performance of an autopsy or operation, may suffice for a rapid and fatal infection. There develops an acute spreading lymphangitis, with enlargement of the epitrochlear and axillary lymph nodes and high fever. Death from general septicæmia may occur, depending on the virulence of the organism and the resistance of the individual. To guard against such sepsis in the post mortem room, the following rule, as recommended by the Pathological Society of Great Britain and Ireland, is to be adopted.

> *" The greatest danger of all and the one chiefly responsible for serious and fatal infections, is pricking the finger. If, during an autopsy, the pathologist should prick himself, however slightly, whether with the knife or on a jagged bone or tooth, even if he is in doubt whether he has been pricked at all, it is his duty to stop at once, remove his gloves and make the wound bleed by digital compression, causing congestion by centrifugal force (swinging the arm), by sucking and putting the hand in hot water. There should not be a minute's delay in carrying out these procedures and the autopsy should be completed by some one else; rest of the injured part is essential."*

Infections of the respiratory tracts:—

Mouth and throat:- Though infrequent, as compared with non-hæmolytic streptococci, the hæmolytic ones may occur as commensals in the throat. Any local pathological process favours their lodgment and it has been found that the natural abode in such cases is the crypts of unhealthy tonsils and adenoids. Such persons who harbour these organisms, may act as *carriers* and serve to disseminate the germs. They may also be responsible for the occurrence of puerperal sepsis and secondary broncho-pneumonia. Hence, it is often urged that persons attending operations and confinement cases should wear masks, not so much for their own safety, but for the protection of the patient. Similarly, a person with a bad throat should not be allowed to enter the ward of infectious diseases without a mask.

Epidemic sore throat: As already mentioned, such cases may often be traceable through milk supply to a dairy farm (*vide* p. 229).

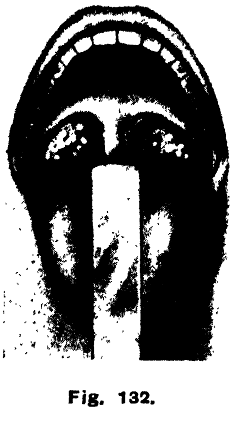


Fig. 132.

Follicular tonsillitis.

In the throat, Str. hæmolyticus produces **follicular tonsillitis** (Fig. 132) and **acute suppurative or peritonsillar abscess (Quinsy)** (Fig. 133). From such places the infection may spread in various directions, e.g. (a) to the lymph glands of the neck, (b) into the accessory nasal air sinuses, (c) by the Eustachian tube into the middle ear and (d) into the general circulation producing a condition of septicæmia or pyæmia.

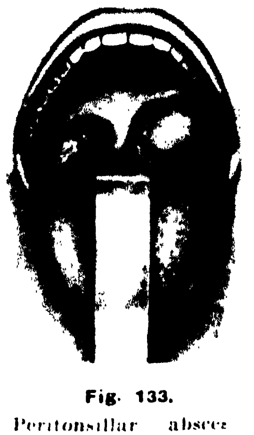


Fig. 133.

Peritonsillar abscess (Quinsy).

Lungs:—The hæmolytic streptococci are mainly responsible for the production of broncho-pneumonia, both primary as well as secondary. The latter often arises as a secondary complication of influenza, measles, diphtheria, scarlet fever, whooping cough and other acute infectious diseases and also occurs in patients suffering from septic processes in the mouth or throat. In these conditions, the organisms are encountered in the throat as secondary invaders and the primary infection only predisposes their invasion. They may spread into the lungs by the bronchial route and by the blood stream.

Empyema, following certain cases of pneumonia, specially after influenza, measles and scarlet fever, is often due to Str. hæmolyticus; in children D. pneumoniæ is the frequent cause.

Scarlet fever:—This disease is very common in temperate climates and is almost unknown in the tropics. A specific type of hæmolytic streptococcus, often called Str. scarlatinæ, is supposed to be the etiological agent. The organisms gain entrance into the throat where they produce their initial lesions and then invasion into the blood may take place, resulting in septicæmia. In these cases, the general symptoms, as well as the rash, kidney and other lesions, are all due to the effects of the toxin. Hence, toxæmia is the main feature of the disease.

A potent toxin has been prepared from this organism and a test has been described by George F. Dick and Gladys Dick (1924), by means of which persons susceptible to scarlet fever can be found out. This is what is known as the Dick test and is carried out as follows:—

DICK TEST:—About 0·2 c.c. of 1 in 1,000 diluted toxin is injected intracutaneously into the forearm. For control purposes, a similar quantity of diluted toxin, heated to 100°C. for 1 hour, is injected into the other forearm, or a little below, into the same forearm. The readings are taken at varying intervals, such as 6 hours, 12 hours and 24 hours. A positive reaction is characterised by the appearance of a bright scarlet erythema which attains its maximum in 24 hours and then gradually disappears in 3 days' time. By this test it has been found that children, between the ages of 6 months and 3 years, are most susceptible.

As toxæmia is the main feature of the disease, recovery is associated with the development of an antitoxin in the blood. Serum from such persons, when injected into an area of skin rash of a patient suffering from scarlet fever, produces blanching of the area injected. This is, therefore, a definite proof of the neutralisation of the toxin by the antitoxin *in vivo* and can be utilised as a means of diagnosis of the disease. This phenomenon was first recognised by Schultz and Charlton and is now known by their name.

SCHULTZ-CHARLTON REACTION:—About 0·2 c.c. of 1 in 10 diluted antitoxic serum is injected intradermally into the newly developed rash of a patient suffering from scarlet fever. Such an injection causes blanching and disappearance of the rash in the area of inoculation within 12 to 24 hours. The reaction is specific, as such a blanching does not occur with other skin rashes.

Infection of the genito-urinary tracts:—

Kidneys:—Acute nephritis very commonly develops in the course of Streptococcal infections. Nephritis which is often a complication of scarlatina, is due to its toxic phenomena; it is also observed that those who suffer from a severe attack of tonsillitis develop nephritis.

Puerperal infection:—The starting point of such an infection may be a perineal wound or a tear in the vagina or the cervix, but, in most cases, it is from the interior of the uterus. After parturition, the placental site is analogous to an open wound and thrombosed sinuses communicate directly with the uterine cavity, thus affording an easy entrance to the invasive bacteria. The commonest organisms are streptococci, the majority being of the hæmolytic type. The infection usually results from an *exogenous* source. The organisms are transmitted, either by the attendants who, at the time of labour, are harbouring virulent strains in their throat and nose or by unclean instruments and dirty hands of nurses and doctors. The possibility of an auto-infection has been raised, but it has been suggested that when such an *endogenous* infection occurs, it is mostly of the non-hæmolytic type.

The lesion most commonly encountered is endometritis. The spread of the infection may take place in the following ways:—(i) By the lymphatics, producing metritis, parametritis, or peritonitis; (ii) by the veins (Fig. 134),

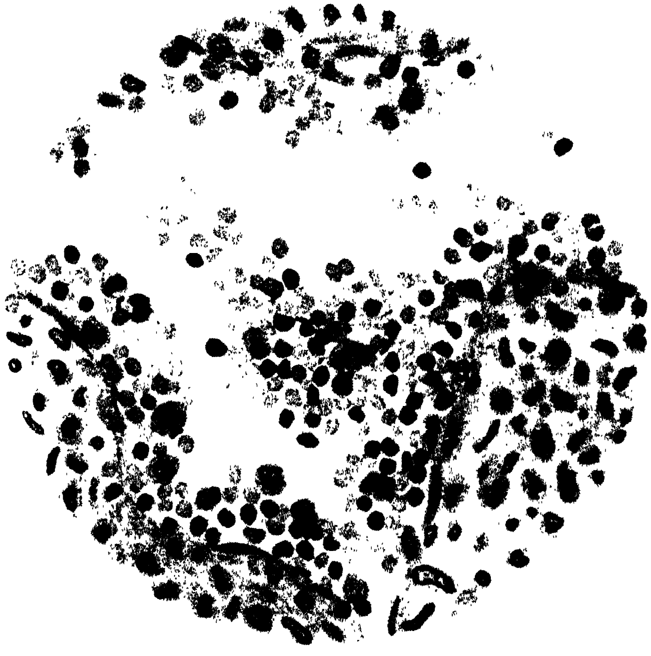


Fig. 134.

Camera-lucida drawing from a micro-section of a puerperal septic uterus, showing streptococci inside an uterine sinus. This beautifully illustrates the origin of streptococcal septicaemia (haemolytic variety) from a septic locus. *Stained by modified Gram-Weigert method.*

leading to septicæmia and pyæmia; or (iii) by the tubes, producing salpingitis, oöphoritis and even peritonitis.

Infection of thrombi may result in septic phlebitis which may spread and involve the internal iliac veins. This may extend through the common iliac to the external iliac veins below, and may produce *phlegmasia alba dolens*.

Testes and Epididymes:—Streptococcal infection of the testes and the epididymes is very common in our country and is often fatal. In most of the cases, filarial infection is also present with or without any active manifestations of the disease. The condition is ushered in by an acute attack of lymphangitis or an inflammation of the testis or epididymis, associated with a sharp rise of temperature, with chill and rigor. The parts become intensely painful and tender. A very marked degree of toxæmia is commonly present. Within 3 or 4 days, all the features of a grave septicæmia supervene and the patient succumbs. In such a case, if a blood culture is done, hæmolytic streptococci may be isolated. When the resistance of the individual is very strong, the acute process passes into a subacute stage, with localisation of the infection and formation of an abscess inside the tunica vaginalis.

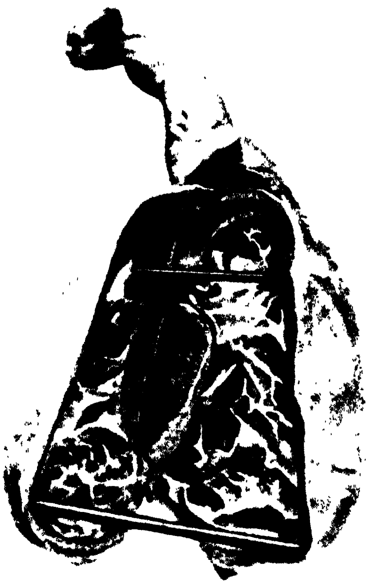


Fig. 135.

Infection with hæmolyticus in Filarial orchitis. Testis which is situated in the centre has been cut open and exposed. The cavity of the tunica vaginalis contains masses of fibrin Micro-filaria bancrofti and Str. hæmolyticus were also found in the exudate. The patient died of streptococcal septicæmia and on *post mortem*, all the signs of acute inflammation were observed extending from the testis along the spermatic cord into the peritoneal cavity.

The gross appearance (Fig. 135) of such a fulminant streptococcal infection is very constant and characteristic. The cavity of the tunica vaginalis is filled up by a fibrinous mass, with varying amounts of slightly cloudy fluid in the dependent part, the surfaces of the tunica showing hæmorrhages. The epididymis and the cord are equally involved and are enormously thickened, being infiltrated with a similar type of exudation. The process is not limited to the scrotum, but is found to extend along the structures of the cord into the abdominal cavity and the acute congestion, hæmorrhage and fibrinopurulent exudate may be traced along the retro-peritoneal lymphatics and blood vessels, on either side of the vertebral column, up to the pillars of the diaphragm. All the lymphatic glands in the inguinal and paravertebral regions are enlarged, swollen and intensely congested.

In the acute stage, the fluid withdrawn from the tunica vaginalis by puncture with a syringe and needle shows a very high albumen content, with few or no cellular exudates. As the process becomes subacute or chronic, large numbers of pus cells appear in it. Micro-filaria are often found in such a fluid. The writers have frequently demonstrated micro-filaria in the hydrocele fluid and adult filaria in the dilated lymphatic varices, as well as, in the lymph nodes. The streptococci which have been isolated from such cases are always of the hæmolytic type and grow well, both under aerobic and anaerobic conditions.

The etiological relationship of this streptococcal infection to the presence of filaria has not yet been understood. It may be that under normal conditions, both the organisms remain in the area in a condition of symbiosis and it is the sudden disturbance of this mutual control which may be responsible for the acute attack. Or it may be that the filarial worms only make the soil suitable for the growth of streptococci, either by the irritant action of the former or their toxin or producing some alteration in the tissue environments by an as yet undetermined factor.

Streptococcal septicæmia and pyæmia:—Sometimes, the localised lesions may result in a generalised infection, the organisms gaining entrance into the blood stream, producing septicæmia or pyæmia. The latter condition is not so common as compared with staphylococcal infections.

Such generalised infections often follow (i) puerperal sepsis, (ii) erysipelas, (iii) scarlet fever and in our country, (iv) acute orchitis and funiculitis. It may also follow wound infections, obtained from injuries received in the post mortem rooms or operation theatres. In most cases of streptococcal septicæmia, the organisms are constantly discharged into the circulating blood from a focus of infection where they are rapidly multiplying. There is high fever, with or without daily remissions, and exacerbations with chills (hectic type of temperature). Blood changes are characterised by a high leucocytosis and destruction of red blood corpuscles producing anæmia. Spleen, in such cases, is enlarged, soft and diffluent in character which is very typical of a septicæmic spleen. Usually, very few localised lesions are found in association with streptococcal septicæmia. There may be a focus in the lung (broncho-pneumonia), joint (arthritis) or heart (endocarditis). The writers have seen a very interesting case of streptococcal septicæmia (hæmolytic strain) supervening on an acute orchitis and funiculitis, with a localised lesion in the pulmonary valve. The gross appearance of such a testis is shown in Fig. 135 (Indian Medical Gazette Oct. 1934).

Endocarditis:—Strep.ococcus hæmolyticus is sometimes the cause of an acute bacterial endocarditis and the character of the lesion is of the ulcerative or malignant type. The vegetations are soft and easily friable and, on being detached, act as emboli which may ultimately lodge in different parts of the body.

Streptococcal meningitis:—This may be a manifestation of general septicæmia, but as already mentioned, it is most commonly due to the extension of an infection from otitis media and mastoiditis.

NOTE:—To isolate the organism from such cases of septicæmia, about 10 c.c. of blood should be withdrawn aseptically from the patient. A portion of it should be left for mouse inoculation while the major portion of it is planted into a flask of glucose broth. When cocci are present only in very small numbers in the blood, animal inoculation method is the best, as blood culture may fail to show any growth.

PATHOGENICITY OF STREPTOCOCCUS VIRIDANS

Compared with the hæmolytic streptococci, these organisms are of a lower virulence and produce a low grade inflammation in which suppuration and tissue destruction are not marked. They are mostly associated with lesions which are subacute or chronic in nature. Thus, endocarditis due to Str. viridans is not so acute and destructive as compared with that produced by the hæmolytic group. Many workers have also observed that a great tendency of these organisms is their selective localisations on certain structures, such as stomach, gall-bladder, endocardium and synovial membranes of joints.

Pathogenic lesions:—

Teeth:—Pyorrhœa alveolaris and apical abscesses are generally produced by this organism. Here, they constantly multiply and produce toxins which are absorbed into the circulation. The bacteria are also discharged into the general blood stream, producing bacteriæmia and a low grade septicæmia. Many obscure inflammatory lesions may ultimately be traced to such apical abscesses produced by these streptococci.

Throat: -Streptococcus viridans causes chronic tonsillitis and pharyngitis. The infection may extend through the nasopharynx and its various connections and may start up inflammatory foci in those places, as already stated.

Appendix:—*Rosenow and Meyer* have emphasised that non-hæmolytic streptococci (Str. viridans) are the organisms most commonly responsible for the production of inflammatory lesions in the appendix (appendicitis).

In this connection, it should be remembered that the chronic inflammatory foci, produced by Str. viridans in the various places mentioned above, serve as areas of *focal sepsis*, from which the bacteria and their toxic products are constantly absorbed into the general circulation, producing serious constitutional disturbances, such as peptic ulcer in the stomach and duodenum, chronic cholecystitis and cholelithiasis, subacute bacterial endocarditis (endocarditis lenta) and chronic arthritis.

Peptic ulcer (Rosenow):—Pathogenesis of peptic ulcer, according to modern conception, has been found to be the result of inflammatory lesions produced by short-chained cocci, the Str. viridans. In the majority of patients suffering from peptic ulcer, some sort of focal sepsis may be found, and it is from these areas that Str. viridans gains entrance into the circulation. Owing to their selective affinity, they are deposited from the blood stream into the submucous coat of the stomach and first part of the duodenum (supra-ampullary portion), where they may set up an acute or chronic inflammation. These may subsequently break down and form small ulcers or erosions. Thus, peptic ulcer is the result of a hæmatogenous infection with short-chained streptococci absorbed from the areas of focal sepsis, specially pyorrhœa alveolaris and apical tooth abscess.

Chronic cholecystitis (*Strawberry gall bladder of Wilkie or cholesterosis of gall bladder of MacCartey*) (Fig. 136).—In the vast majority of cases of chronic cholecystitis, these short-chained streptococci, producing smooth non-hæmolytic colonies on blood-agar, are found to be responsible. Cholecystitis has been considered to be a blood-borne infection by Str. viridans, the organisms reaching the submucous coat of the gall bladder through the cystic artery. From this intramural infection, the organisms infect the bile, but owing to the antiseptic power of the latter, the growth of streptococcus is inhibited. Hence, culture from bile is found to be sterile, although growth is obtained from the muscular and submucous layers. In over 80 per cent. of the cases, isolation of the organisms is also possible from the cystic lymph gland which may be enlarged to the size of a small bean.

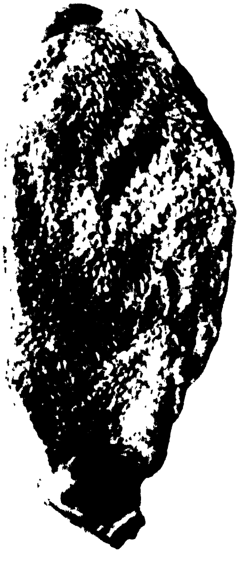


Fig. 136.

Strawberry gall bladder with a solitary stone. The white specks represent the deposit of cholesterol ester in the submucous coat.

Heart:—The lesion most commonly encountered is that of the valvular endocardium, producing a condition called subacute bacterial endocarditis or endocarditis lenta. In this condition, the organism is said to have been derived from an apical tooth abscess (Fig. 137). Str. viridans usually tends to infect a cardiac valve which has already been damaged by disease or otherwise. In this affection, besides the changes in the cardiac valves, petechial hæmorrhages occur under the skin. This condition is the result of damage to the capillary endothelium. In the kidney, the glomerular elements are affected, due to the lodgment of minute emboli from the cardiac vegetations. Such a lesion of the kidney is known as *embolic focal glomerular nephritis* and is characterised by the formation of numerous small hæmorrhagic spots (infarcts) in the organ, for which the latter is often described as *flea-bitten kidney* (Fig. 138). Another point to be remembered is that these lesions are non-suppurative in character. In such a condition, the urine shows albumen and red blood cells. This is very constant and is of great diagnostic help in this form of endocarditis.

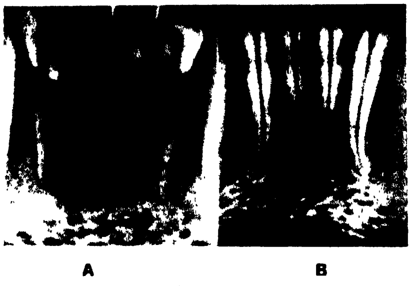


Fig. 137.

Skiagram of apical tooth abscess (A) compared with the normal (B).

Relation of Streptococcus viridans to acute rheumatic infection:—*Poynton and Paine* (1900) and later *Rosenow* isolated Str. viridans from the joint lesions of rheumatic cases. The latter has also been able to reproduce the disease in animals (rabbit) by injecting these organisms, isolated from the joint lesions of rheumatic cases. Others are sceptic about it and consider them as causal commensals. Hence the question is still open, but it is to be remembered that tonsillitis, arthritis and cardiac lesion are intimately associated with one another and with the presence of these organisms.

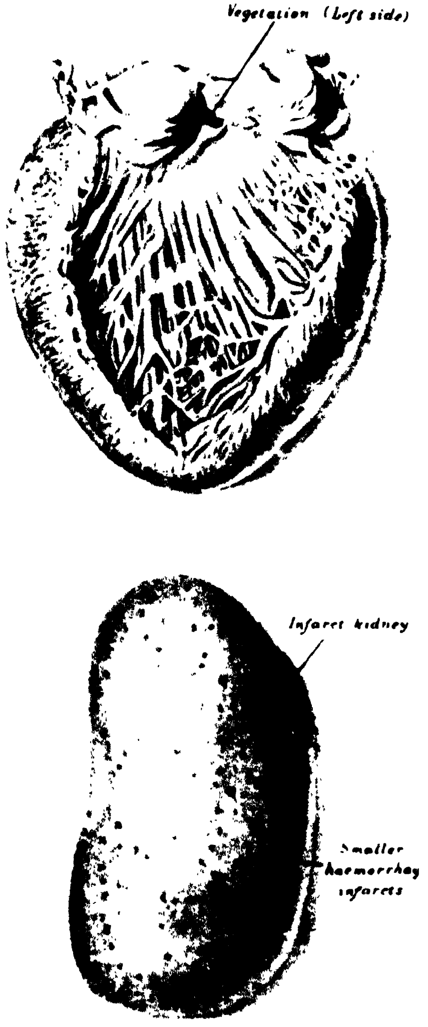


Fig. 138.
Subacute bacterial endocarditis with multiple haemorrhagic infarcts in the kidney (flea-bitten kidney).

PATHOGENICITY OF STREPTOCOCCUS PNEUMONIÆ:—As already mentioned, this strain belongs to type III D. pneumoniæ; hence, its pathogenic lesions will be the same as those of the latter.

PATHOGENICITY OF STREPTOCOCCUS FÆCALIS (ENTEROCOCCUS):—Streptococcus fæcalis is probably a natural inhabitant of the intestinal tract. It is a saprophytic microbe and is usually non-pathogenic, but if conditions are suitable and environments are altered, they may become pathogenic. For instance, in cases of typhoid fever, in which ulceration of the bowels takes place, these cocci might get into the blood stream, producing an additional bacteriæmia. Under such circumstances, they may be isolated from the blood of the patient. Whether they have any recognisable effect on the course and prognosis of the primary disease, is a matter which has not yet been settled. In this connection, it should be noted that a series of observations was recorded by *Shanks, Khan and Tribedi* (1930) in which a bacteriæmia with Str. fæcalis (Enterococcus) occurred as a complication of typhoid fever. They concluded from their observations that the organism was a temporary, and relatively innocuous, invader of the blood stream. Numerous cases have also been recorded by various writers (*Andrewes and Horder*, 1906; *Besson*, 1920), in which they have observed bacteriæmia with Str. fæcalis, either secondarily to another infection or primar

THERAPEUTIC CONSIDERATIONS

Streptococcus infection in man, as a rule, does not, to any marked degree, confer immunity against these bacteria. Thus erysipelas may occur repeatedly in the same individual and even in the same anatomical locality. When a streptococcal infection is overcome, it is not due to the development of any bactericidal power or anti-toxic property of the serum (except in scarlet fever), but is the result of cellular defence. Of the cells, the polymorphonuclear leucocytes are hardly responsible in preventing the spread and it has been shown that an exudate rich in polymorphonuclear leucocytes could not arrest the extension of streptococcal infection. The cells which are concerned in this process are the wandering mononuclear cells, belonging to the reticulo-endothelial system. These mobilise at the site of infection, and it is after such a localisation, that the polymorphonuclear leucocytes, aided by the opsonic substances, can dispose of the streptococci.

Serum therapy:—An antiserum (antibacterial) has been prepared against streptococci and has been used in the treatment of streptococcal infections without much success. This is so because the serum does not contain any antitoxin which can neutralise the effects of streptococcal toxins, nor does it possess any bactericidal property. It contains substances which promote phagocytosis. The beneficial results that have been met with in some cases by the use of such sera, are not due to any specific properties, but are the result of stimulation of the reticulo-endothelial system by non-specific serum proteins.

Lately, a potent antitoxic serum (1 c.c. neutralising at least 1,000 skin-test doses) has been prepared against the haemolytic strain of streptococci causing scarlet fever and is being used extensively in the treatment of that disease with marked success. Such a serum can neutralise the toxic effects of the organisms. As acute toxaemia is a feature of most of the fatal cases of streptococcal septicaemia, this anti-scarlatinal serum may produce beneficial effect in severe cases of infection with Str. haemolyticus, other than scarlet fever.

Vaccine therapy:—Specific vaccines are often used as a therapeutic measure and when injected in gradually increasing doses, undoubtedly raise the opsonic power of the serum. They may be used as curative agents in many chronic streptococcal infections, where it is always preferable to use autogenous vaccines. As a prophylactic measure, it is often combined with catarrhal and pneumococcal vaccines. In *scarlet fever,* however, an active immunity can be produced by injecting gradually increasing doses of scarlatinal toxin, which probably lasts for several years. This method is used as a prophylaxis for scarlet fever and is carried out by giving 3 weekly subcutaneous injections of scarlatinal toxin, diluted in saline, so that 1 c.c. contains 500 'skin-test doses.' ('Skin-test dose' is the unit of scarlatinal toxin and is defined as the smallest amount of toxin which is sufficient to cause an erythematous reaction, 1 cm. in diameter, in the skin of susceptible persons, within 24 hours). The dose varies from 0·2 to 2 c.c., in the case of children under 12 years, the third dose should never exceed 0·5 c.c.

ANAEROBIC STREPTOCOCCI

Veillon (1893) first described these organisms under the name of Micrococcus fœtidus, owing to the production of a foul odour in culture. At present, the literature contains many references about the anaerobic streptococci and several varieties have been isolated from various suppurative and gangrenous conditions of the urogenital tract, appendix, lungs and serous membranes. Normally, these are frequently found in the vagina and on other mucous membranes of the body. They are chained cocci, sometimes appearing in pairs. They stain well with ordinary aniline dyes and are Gram-positive. They are strictly anaerobes, as mere traces of oxygen are sufficient to inhibit their growth. They grow best at the body temperature. Neutral or slightly alkaline reaction of the media is favourable for their growth. For cultivation, the anaerobic jar of Fildes and McIntosh may be used. The organisms grow in all ordinary media, preferably those containing glucose, serum or blood. Deep-glucose agar has been extensively used for their cultivation. Regarding biochemical reactions, they possess no hæmolytic properties, their fermentation reactions are very variable and they produce gas and putrefactive odour in culture medium. Experimental inoculation of cultures into laboratory animals generally proves negative. As regards their pathogenic rôle in human beings, some strain plays an important part in the production of various septic conditions, such as adnexal suppuration, septic thrombo-phlebitis, and secondary lung abscesses, following puerperal sepsis. They have also been found in pulmonary gangrene, gangrenous appendicitis and septic endocarditis following septic abortion and, in particular, they determine the putrid character of the lesions concerned.

CHAPTER XX

DIPLOCOCCUS PNEUMONIÆ

(Pneumococcus; Streptococcus lanceolatus)

This is the micro-organism which causes lobar pneumonia and broncho-pneumonia. It is one of the bacterial species in which the phenomenon of autolysis is very pronounced and constant. These are cocci arranged in pairs. Here the daughter cells after cell-division remain united for a short period and then separate before any further division takes place.

HISTORICAL:—A capsulated diplococcus causing septicaemia in rabbits was first described by *Pasteur* (1881) but he did not, in any way, associate this micro-organism with lobar pneumonia. *Talamon* (1883) first accurately described the Diplococcus lanceolatus. His work was confirmed and supplemented by *Albert Fraenkel* and *Weichselbaum* who published their results independently in 1886 and established, beyond question, the importance of D. pneumoniæ as the etiological factor in a large majority of the cases of lobar pneumonia.

HABITAT:—Only in 20 per cent. of the cases, they are found as normal inhabitants in the upper respiratory tracts of man, and of these, 50 per cent. belong to Type IV (Group IV).

MORPHOLOGY (Fig. 130): This is best studied when the organisms grow inside the animal body or in culture media containing serum, blood or any body fluid.

Shape:—The cocci are lance-shaped with their bases apposed to each other, and hence they are called Diplococcus or Streptococcus lanceolatus. They may be oval also.

Arrangement:—It is a diplococcus. In fluid culture media, two or more pairs may remain attached forming a short chain. Thus, one may mistake it for a Streptococcus.

Capsule formation:—It possesses a well defined capsule which appears as a clear halo when not stained by special methods. It is important to remember some points about the capsules of D. pneumoniæ:

(i) The capsule reaches its fullest development in the animal tissues and the organism tends to lose it in an artificial culture.

(ii) Its presence is distinctly associated with the virulence of the organism.

(iii) It behaves as an antigen and gives rise to the formation of an antibody, bacteriotropin (immune opsonin)

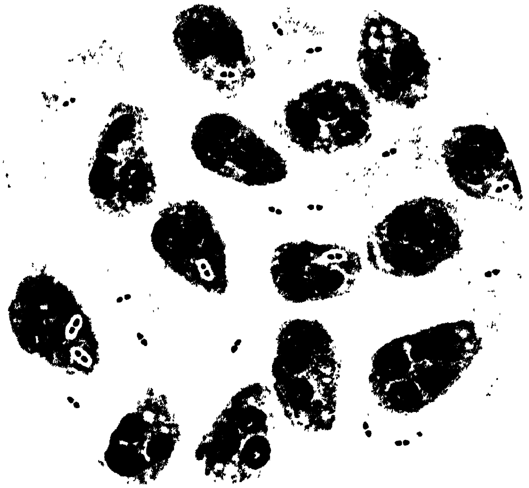


Fig. 139.

A smear of pus showing the morphological characters of *Diplococcus pneumoniæ*. Note the lance-shaped diplococci surrounded by a clear halo, representing the capsule. Some of the cocci have been phagocytosed by the polymorphonuclear leucocytes.

(iv) The capsular substance contains a polysaccharide which is responsible for the type-specificity, and its chemical composition varies in different types but is constant for a particular type.

(v) Transformation of colonies from smooth to rough forms is associated with the disappearance of the capsule (Fig. 140), loss of virulence and type-specificity of the organisms.

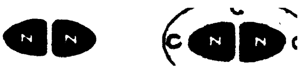


Fig. 140.

Diagram representing the rough non capsulated and [illegible] *D.* *p* [illegible] R rough [illegible] form. N [illegible] so[illegible] hydrate

STAINING REACTIONS:—It stains readily with the ordinary aniline dyes. It may be rapidly and conveniently stained with warm dilute carbol fuchsin. With Gram's method of staining, it is *Gram-positive*. Special methods of staining are required to demonstrate the presence of the capsule (see page 27).

CULTURAL CHARACTERS:—It is aerobic and requires an enriched medium for its cultivation. Presence of glucose in the medium promotes growth at first but the organisms are soon killed by the resulting acidity. The cocci tend to die out very quickly, specially in liquid media.

In broth, the organism grows, producing moderate turbidity. The culture becomes clear in 48 hours due to rapid autolysis of the cocci in fluid medium.

In nutrient agar, D. pneumoniæ generally refuses to grow In agar with blood, serum, hydrocele fluid or ascitic fluid (p. 60), the organisms grow very well. ***Colonies*** are small, delicate, discrete, transparent, moist and flat. In *blood agar*, the colonies produce a dark green colour like Str. viridans, due to the formation of methæmoglobin (Fig. 141). There may be a zone of partial hæmolysis (*a* type of hæmolysis).

Colony variation:—Two types of colonies are met with in D. pneumoniæ. The one with a smooth, shiny surface (*smooth type*) and the other with a slightly granular surface (*rough type*). The former is usually more virulent than the latter.

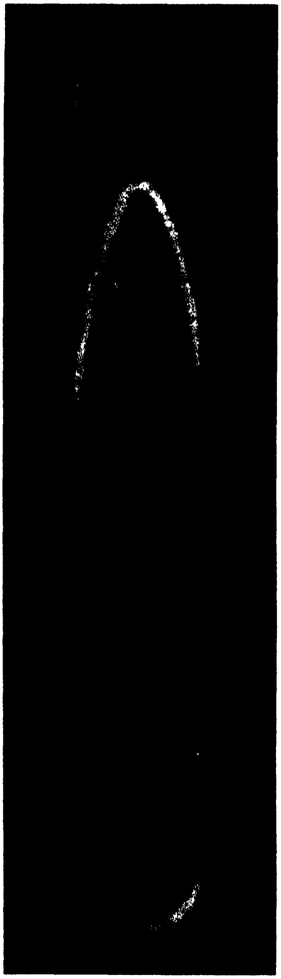


Fig. 141.

Colonies of *D. pneumoniæ* on blood agar, showing the characteristic flat colonies surrounded by a green halo.

BIOCHEMICAL REACTIONS:—

(i) ***Fermentation Reactions:***—D. pneumoniæ ferments the following sugars with the production of lactic acid:—

Mono-saccharides—Glucose, levulose and galactose.

Di-saccharides—Lactose, maltose and saccharose.

Tri-saccharides—Raffinose.

Poly-saccharides—*Inulin*, dextrin.

The glucoside, salicin, is not fermented.

For the study of sugar fermentation, Hiss's serum water media (p. 72) should be used.

(ii) ***Oxidation power:*** The conversion of hæmoglobin into methæmoglobin is an example of oxidation process. The methæmoglobin is only an isomer of oxyhæmoglobin and is formed by the oxidation of the iron in the latter, converting it from the ferrous into the ferric state. This change occurs only under aerobic conditions.

McLeod and Gordon have found that D. pneumoniæ produces hydrogen peroxide in cultures under conditions of good aeration. As no catalase is produced by the organisms, the peroxide thus formed is not destroyed by them and hence, it may accumulate to such a concentration as to cause death of the organisms. This is one of the factors which will be taken into account to explain crisis in lobar pneumonia and death of D. pneumoniæ in the consolidated areas.

(iii) ***Solubility in bile:*** It has been found that bile exerts a specific bacteriolytic effect on D. pneumoniæ and this serves as one of the important tests for the differentiation of the organism from the Streptococcus of the viridans group. In this connection, it should be noted that the bile salt acts simply by hastening the usual autolytic process.

Technique of testing bile solubility:—To produce lysis, sterile ox-bile is added to a broth culture of D. pneumoniæ, in a dilution of 1 in 10. The culture becomes clear in 10 minutes. The substances most actively concerned in the reaction are sodium salts of bile. Hence, instead of using bile which varies in action according to the quantity of the salt present, it is better to use a solution of pure sodium taurocholate for the purpose. To carry out the test, a 10 per cent. solution may be made and kept in stock, as it keeps indefinitely, and 0·1 c.c. of this solution is added to a 5 c.c. broth culture or suspension of D. pneumoniæ, which will become clear in about 10 minutes. The test may also be done on a slide either by mixing a little fluid culture or by emulsifying a little growth from blood agar with a little bile salt solution and watching under the high power of the microscope, when the cocci will be seen to undergo lysis.

VIABILITY: The vitality of D. pneumoniæ cannot be maintained in artificial media for a long time. *In fluid media,* the organisms die out rapidly if kept in the incubator or at room temperature. Rapid autolysis, however, takes place only after the first 12 hours. *On solid media,* they survive for a considerable period if kept in the ice-chest or in sealed tubes. Hence the necessity for frequent subcultures to keep the strain alive. In glucose media, the organisms die out very quickly owing to the increasing production of acid.

If rapidly and thoroughly dried, the organisms can survive for long periods *in animal tissues.* Neufeld has succeeded in keeping D. pneumoniæ alive and virulent by aseptically taking out the spleens of mice dead of pneumococcus infection and preserving them in a Petri dish, in a desiccator, in a cool dark place. In this way, the organisms can be cultivated from the spleen and will be found virulent for longer periods than when preserved in culture media. The best way to obtain the organisms in pure culture from such a dried material is by injecting a suspension of the desiccated spleen in broth into a white mouse and recovering them from the heart's blood.

In sputum, the viability of D. pneumoniæ seems to exceed that in culture. In dried sputum, when protected from light, the organisms remain alive for 1 to 4 months. When exposed to diffuse light at room temperature, they survive for 19 days. Recently, observations have been made upon the pneumococcus viability in finely divided sputum, i.e., in a condition in which transmission by inhalation would be possible. It has been found that D. pneumoniæ can survive only for about one to one and a half hours under ordinary conditions of light and temperature.

Heat is very injurious to the life of the organisms which are readily killed when exposed to a temperature of 60°C. for 20 to 30 minutes.

TOXIC PRODUCTS OF D. PNEUMONIÆ:—So far no specific pneumococcal toxins have been isolated or identified. Poisonous substances are no doubt liberated by the death and disintegration of the organism from the split products of bacterial protein, but they fail to explain the abrupt onset of acute fever and toxæmia. Though the organisms do not produce any definite demonstrable exotoxin, it will be shown later that they secrete a specific soluble substance during their growth, which diffuses into the surrounding medium and is found in all body fluids. Such specific soluble substance (S. S. S.) may be responsible for the intoxication in lobar pneumonia but there is no direct evidence that it is a general toxin. This substance *can act as aggressin* and is definitely related to the virulence of the organism. The latter property does not depend on any toxin but on its ability to multiply in the tissues. Further it should be noted that this specific soluble substance is intimately associated with the capsular material which determines the virulence of D. pneumoniæ and prevents their phagocytosis. When the specific soluble substances are liberated in large amounts, they neutralise the antibodies circulating in the blood and thus the substance on the surface of the organism remains intact, which prevents phagocytosis. This favours multiplication of the bacteria and it is known that cases with marked bacteriaemia are invariably fatal.

The organisms also produce haemolysins which bring about lysis of red blood corpuscles (not haemodigestion). Jaundice in some cases of pneumonia and pneumococcal infection may be explained by the action of this haemolytic toxin on the blood.

SEROLOGICAL REACTION:– No antitoxin has yet been found in the immune serum or in the serum of patients who have recovered from an attack of pneumonia. The substances which have been definitely demonstrated in the antisera are *specific agglutinins and precipitins* both of which appear to be identical substances. An immune substance of the nature of *opsonin (bacteriotropin)* is also developed. The latter neutralises the specific soluble substance on the surface of the bacteria, thereby promoting phagocytosis and intracellular lysis. The beneficial result that ensues as a result of serum therapy is due to this bacteriotropic effect. Thus, it will be seen that anti-pneumococcal serum may possess the following properties: (i) agglutination reaction, (ii) precipitin reaction and (iii) opsonic action. The first two are utilised in the diagnosis of pneumococcal infection and in finding out the various types of the organism, as they are found to be type-specific (*vide infra*). For the latter property, the serum is used therapeutically and it has been further observed that such a specific effect of the serum, i.e., the protective power, is only present in Type I antisera and to a less extent in Type II antisera.

TPYES OF DIPLOCOCCUS PNEUMONIÆ

It is a very interesting fact that although all D. pneumoniæ have identical morphological and cultural characteristics, they cannot be classified as a single species. On the contrary, there are very sharp serological differentiations. For instance, if they form only one species, all the strains from different cases should be agglutinated by one and the same immune serum and the same one antiserum should protect mice when inoculated with the various strains isolated from different cases. But this is not found to be true. The study of this interesting subject was started and carried on extensively in America and, as a result, it has been found that D. pneumoniæ fell into 4 different classes, designated as Types I, II, III and IV. These have been determined by cross-agglutination and absorption tests.

Types I and II:—Morphologically and culturally they are typical D. pneumoniæ. Potent antiserum has been obtained for each of them, specially for Type I. They are responsible for the production of greater number of cases of pneumonia, although the case mortality is not very high. These are the so-called "epidemic types" and are responsible for the production of 60 per cent. of cases of lobar pneumonia.

Type III:—It is also called Streptococcus mucosus (Schottmüller) and is recognised by its characteristic cultural property, producing mucoid colonies. It is very virulent and produces a very dangerous type of pneumonia and accounts for 15 per cent. of cases investigated. It does not give rise to any protective serum.

Type IV (Group IV): —This includes a heterogeneous group, comprising all of the D. pneumoniæ which can not be serologically placed into the above three types. They do not produce serious lesions and prognosis is not so bad. No antiserum has been prepared. These are normally present in the nasopharynx of human beings (10 per cent.).

Table showing the incidence and mortality of different types

	Incid		Specific antiserum.
Type I and Type II.	60 per cent.; Type I is much more common.	25 to 30 per cent.	Therapeutic antisera are available for both types but they are much more potent for Type I.
Type III.	15 per cent.	50 per cent.	No therapeutic antiserum.
Type IV.	25 per cent.	12 per cent.	No therapeutic antiserum.

Species and type specificity: Every coccus belonging to this group has been found to consist of two structures, viz., the bacterial body and the capsule, and these have been subjected to a very critical scientific study. The *bacterial body* consists of a material which is nucleoprotein in nature and is the same in all the types of the organism (Fig. 142). It gives a positive precipitation reaction with all the type antisera and cannot, therefore, be type-specific, although it is species-specific. This bacterial protein is highly

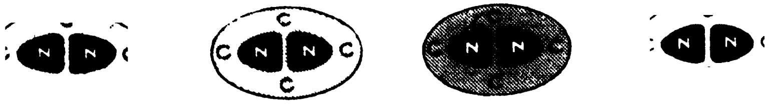


Fig. 142.

Diagrammatic representation of the structure of various types of *D. pneumoniæ*. The somatic antigen N is the same in all types, whereas the capsular antigen C varies in each type, as shown by different grades of shading.

antigenic, being capable of provoking a strong antibody response in animals. It appears in solution in fluid cultures rather late, i.e., only after the organisms have begun to undergo autolysis which takes place after 12 hours.

The *capsular part* of the organism, on the other hand, consists of a carbohydrate material, polysaccharide in nature, and varies in its chemical composition in the different types, but remaining strictly constant for a particular type (*vide* Fig. 141). To this carbohydrate capsular moiety, the D. pneumoniæ owes its type-specificity. Dochez and Avery (1917) have found that this specific polysaccharide substance makes its appearance in a pneumococcal broth culture at a very early stage and reaches its maximum concentration in about 6 to 8 hours, i.e., long before death and autolysis of the organisms have begun and therefore, this substance is not an autolytic but a secretory product elaborated by the vital activities of the bacterial cells. This specific soluble substance (S.S.S.) is present not only in culture fluids but also in the animal body, in the sputum, urine, pus and other pneumococcal exudates in an infected person. This specific soluble substance (polysaccharide) gives a precipitin reaction with the homologous immune serum and thus can be used as a method for type determination. It has already been remarked that this S.S.S. acts like aggressin and is definitely related to the virulence of the organisms and its progressive elimination in the urine is significant from the prognostic standpoint. The increased amount in the urine is proportionate to the severity of the disease. A further investigation of the property of these polysaccharides has proved that they are completely devoid of any antigenic power and are thus incapable of provoking any antibody response in the animal. But although these carbohydrates are antigenically inert, by themselves, they are quite capable of behaving as good antigens when they are used in combination with the bacterial protein, as found naturally in the intact D. pneumoniæ. Such a union of the carbohydrate (polysaccharide) with the pneumococcal protein (nucleoprotein) confers upon the former its power to act as an antigen and as soon as the link is broken, it is deprived of this function. These type-specific polysaccharides are, therefore, classed as *bacterial haptenes or partial antigens*. The name "haptenes" was introduced by Landsteiner (1919) to a class of bacterial substances which, while they react specifically with antibodies, are, by themselves, devoid of any antigenic capacity. When, however, they are linked with the bacterial protein, they become highly antigenic.

VIRULENCE OF D. PNEUMONIÆ: The virulence of D. pneumoniæ varies considerably. It may be completely lost in artificial cultures, specially in the absence of body fluids in the media. It can be exalted by passage through susceptible animals. High virulence is associated with vigorous capsular development. Rough forms are generally devoid of a capsule and are therefore avirulent or very much less so.

ANIMAL INOCULATION: -D. pneumoniæ is pathogenic to various animals. The susceptibility, however, varies with different species. The rabbits and specially the *white mice* are very susceptible. The guinea-pig, the rat, the dog and the sheep are fairly resistant and the birds are practically immune. It should be noted that the disease produced in susceptible animals is not pneumonia but a general spticæmia. *Blake and Cecil* have been able to produce, by intratracheal injection of virulent D. pneumoniæ in monkeys, typical lobar pneumonia as in human beings, recovery taking place by crisis.

PATHOGENICITY

IN ANIMALS: Spontaneous pneumococcal infection amongst animals is neither very important nor very frequent. Scattered references are, however, available in the literature about such infection in laboratory animals that are commonly used for experimental purposes. *Blake and Cecil* (1920) have observed the development of spontaneous pneumonia amongst monkeys which were kept in captivity in overcrowded places. The peculiar feature of all these infections is that they are usually caused by Group IV. D. pneumoniæ.

IN HUMAN SUBJECTS:—It has already been mentioned that D. pneumoniæ are found normally in the mouth and naso-pharynx in about 20 per cent. of healthy individuals. Out of these persons, about 50 per cent. harbour organisms belonging to Group IV, which are least virulent and the rest harbour organisms belonging to fixed and virulent types (Types I, II & III), being the so-called carriers. It has been remarked by American observers that persons coming in contact with Types I and II pneumonia cases harbour the organisms in their throats in 40 per cent. of cases for an average period of 23 days and they may suffer from pneumonia on account of this. It has been further observed that D. pneumoniæ survive in the throats of convalescent patients for as long as 60 days from the onset of the disease.

The normal lungs and bronchi have, however, been found to be sterile. The question arises that when an individual develops acute lobar pneumonia, whether it is the result of an exogenous infection or an auto-infection from the organisms harboured by him. Although there is a possibility of the former type of infection, specially during intimate contact with actual cases or convalescents, there is also every probability of an auto-infection. The exact method by which D. pneumoniæ normally inhabiting the throat becomes suddenly pathogenic and causes pneumonia is not definitely known. But there are certain factors which undoubtedly predispose an individual to a pneumococcal infection of his lungs. These are trauma, exposure to cold, catarrh due to common colds, chronic alcoholism and chronic tropical splenomegalic conditions due to Leishmaniasis, etc.

The **mode of entry** of the organisms into the lungs is a very debatable point and there is a great deal of discussion concerning the *route* by which the organisms reach the lungs. There are three possible ways by which the organisms may spread into the lungs. These are as follows:—

(i) *Extension along the surface of the air passages (Bronchogenous):*—A direct extension of the inflammation from the upper respiratory tract is not possible since the remarkable lobar distribution of the lesion is against such bronchogenic extension.

(ii) *Extension by way of the lymphatics:*—The organisms may penetrate the mucous membrane of the trachea or bronchi and spread by way of the lymphatics. The pulmonary alveoli are thus reached from the interstitial tissues and not from the bronchioles and the [illegible] extends peripherally from the hilus. The great upholders of this method of spread are *Blake and Cecil* who, in 1920, deduced their conclusions from their experimental works on monkeys. They do not consider that septicæmia is a necessary precursor to the development of pneumonia and, when it does occur, it may be regarded as an overflow phenomenon.

(iii) *Extension by way of the blood stream (Hæmatogenous):* According to this view, the organisms enter the blood stream from the upper respiratory tracts, producing a primary septicæmia with ultimate localisation in the lungs.

At present it is very difficult to decide by which route the organisms spread into the lungs. Although Blake and Cecil have brought forward convincing proofs of lymphatic spread, yet it cannot be taken as finally settled. We are, however, particularly in favour of maintaining the last two views.

Primary lesion in the respiratory tracts:—

In the *UPPER RESPIRATORY TRACT* which is the portal of entry and the normal abode of the organisms, mild inflammatory conditions may be produced. Usually a catarrh of the nasopharynx, leading to sore throat, results. As mentioned on previous occasions, the infection from the nasopharynx may then spread (Fig. 143) into the nose *(rhinitis and its*

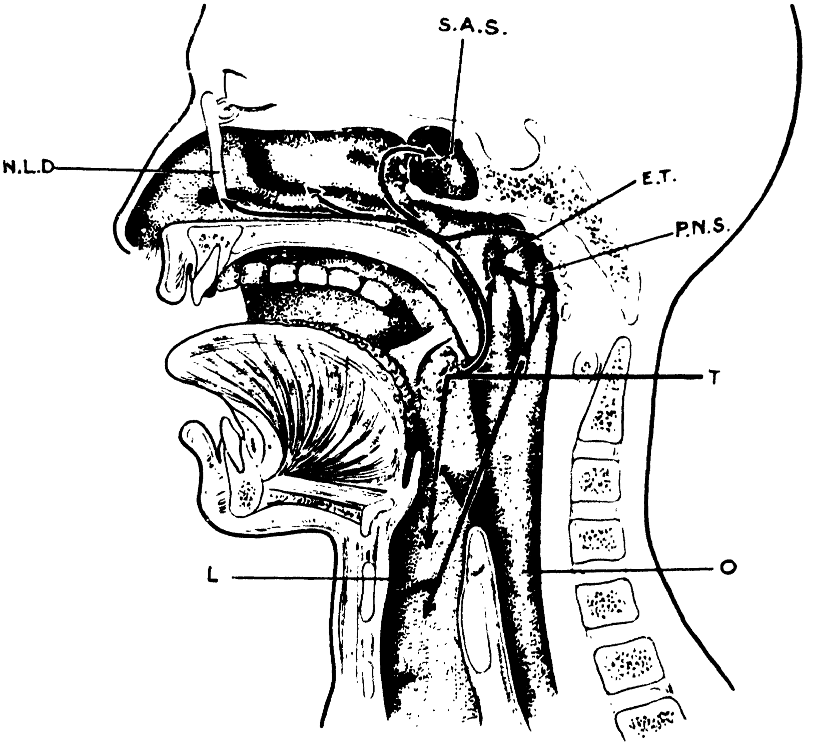


Fig. 143.

Diagram showing the extension of pneumococcal infection, as indicated by arrows, in the upper respiratory tract from tonsillitis and pharyngitis T—tonsil, P.N.S. post-nasal space, L larynx, E.T. opening of Eustachian tube, S.A.S.—sphenoidal air sinus, N.L.D.- naso-lachrymal duct, O œsophagus.

The arrow in the middle meatus indicates the extension of infection into the maxillary, frontal and ethmoidal air sinuses.

complications), middle ear via Eustachian tube *(otitis media with its complications* Fig. 144) and the lower respiratory tracts *(laryngitis, bronchitis)*. An important complication of the extension of inflammation of the upper respiratory tract is the ocular lesions, such as *dacryo-cystitis and corneal ulcers.*

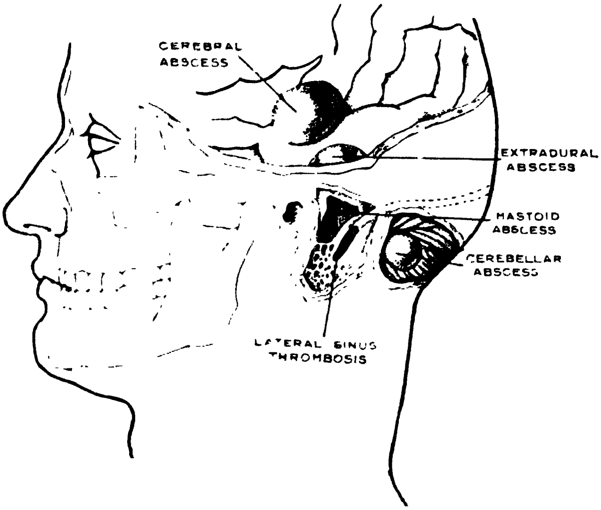


Fig. 144.

Otitis media and its complications

In the *LOWER RESPIRATORY TRACTS* the organisms produce an acute infectious disease, lobar pneumonia, with which the name of the organism is so intimately associated and which is due to D. over 90 per cent. of the cases.

Lobar Pneumonia: The disease is commonest in young adults and is characterised by an inflammatory condition of the lung parenchyma, producing consolidation of the organ. This may affect large areas of the organ, involving uniformly one or more lobes. The affected lung is found to be much increased in volume, feels solid and heavier than normal (pieces sinking in water). It may look either chocolate red or greyish white in colour, according to the stage of the disease in which the examination is made. On section, the substance of the lung feels solid and looks granular, resembling very closely a slice of liver and it is for this reason that this process of consolidation is commonly called "hepatisation" and the affected part of the lung is said to be "hepatised." A very characteristic feature is the sharp line of demarcation between the healthy and the affected parts and this line of separation is commonly found to follow the borders of one or more lobes (Fig. 145). It is owing to such a distribution of the lesions that the disease has derived its name, "lobar pneumonia." The surface of such a lung is raised above the level of that of the healthy one and one often finds indentations of ribs marked on it. The pleura may show various grades of

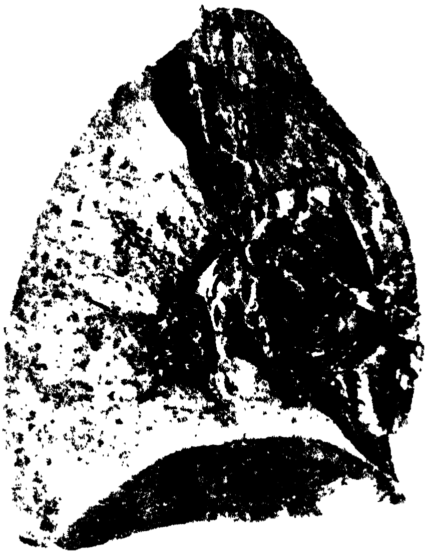


Fig. 145.

Acute lobar pneumonia, showing the gross appearance of the lung in the stage of grey hepatisation. Note the sharp line of demarcation between the healthy and the affected parts.

inflammation and may be covered with a very thick, tough, fibrino-purulent exudate which may penetrate into the interlobar septum.

When such a lung is examined *under the microscope*, the alveoli are found to be filled up with an inflammatory exudate, consisting chiefly of polymorphonuclear leucocytes and a varying number of red blood cells and D. pneumoniæ entangled in a network of fibrin (Fig. 146). In the early stages of the disease, the red cells and fibrin preponderate and, for this reason, the condition is commonly designated as "*red hepatisation*," but in the later stages, they are almost completely replaced by numerous leucocytes, conferring greyish

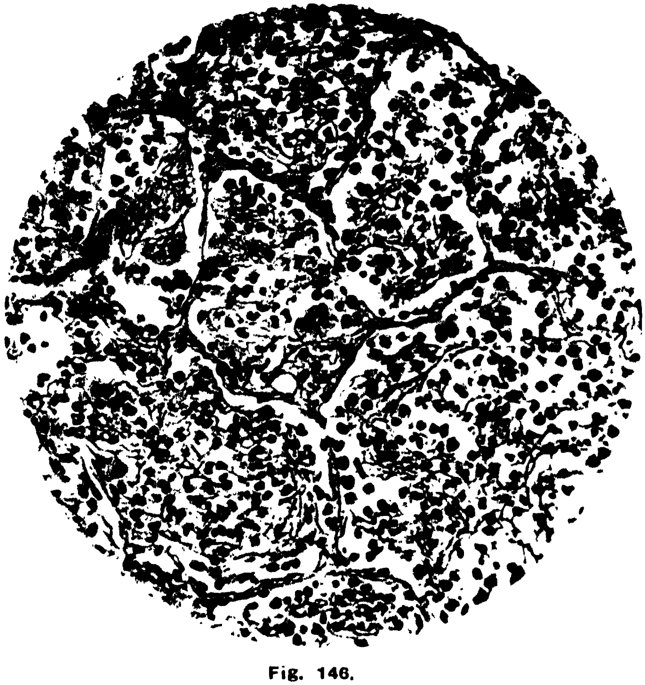


Fig. 146.

Photomicrograph of a lung in the stage of grey hepatisation Note the alveoli are filled with a large number of polymorphonuclear leucocytes with very little fibrin and a few red cells.

appearance to the affected part which is said to be in a stage of *"grey hepatisation."*

Lobar pneumonia is a self limited disease, runs a short course and, in favourable cases, ends by crisis, the explanation of which is given later. Healing is uneventful and it is often impossible to detect any signs of the disease in the lung in an autopsy examination done later on. In unfavourable cases, the organisms again enter the circulation, producing secondary septicæmia which may give rise to various complications, such as meningitis, pericarditis, endocarditis, peritonitis, empyema, arthritis or, even in some cases, pyæmia. All these lesions are characterised by a fibrino-purulent inflammation. A very interesting case was observed by the writers in which the following lesions were found in the same case—lung in the stage of grey hepatisation, empyema, fibrino-purulent meningitis and ulcerative endocarditis of the aortic valve. The endocardial lesion of this case is shown in Fig. 148.

Broncho-pneumonia: D. pneumoniæ is the commonest cause of primary broncho-pneumonia in babies below 2 years of age and also in old persons. It may also be the cause of secondary broncho-pneumonia which arises as a complication of many acute infectious diseases. The lesion is characterised by patchy consolidations, the inflammation starting from the respiratory bronchioles and extending into the neighbouring alveoli.

Other lesions:—

D. pneumoniæ may invade all the serous membranes of our body, which appear to be particularly susceptible to such an infection. As it is a pyogenic organism, its localisation always leads to pus formation.

Meningitis: Primary pneumococcal meningitis may be a manifestation of general septicæmia and may occur without lobar pneumonia. Secondary pneumococcal meningitis may result as a complication of lobar pneumonia or from extension of inflammation from the middle ear. The lesion is characterised by the formation of a thick fibrino-purulent exudate which is of a greenish colour and distributed more conspicuously over the vertex, as in N. meningitidis. The cerebrospinal fluid is under pressure, greenish in colour, thick and purulent. The protein content is increased considerably and Lange's colloidal gold reaction gives the typical meningitic curve. There is a marked diminution of sugar with an increase of the pH. Microscopical examination reveals the presence of numerous polymorphonuclear leucocytes and Gram-positive capsulated diplococci, most of which are free and extra-cellular.

Pericarditis (purulent): The infection may be (a) hæmatogenous or (b) by direct extension from the lungs, though the latter is not the usual rule, but may sometimes occur by a process of continuity and contiguity. Both the parietal and visceral layers of the pericardium are acutely inflamed and covered over with a thick fibrino-purulent exudate (Fig. 147). The latter is most marked over the surface of the heart which is completely covered over with the shaggy mass. The pericardial cavity may contain a varying amount of thick, creamy, greenish pus mixed with flakes of fibrin.

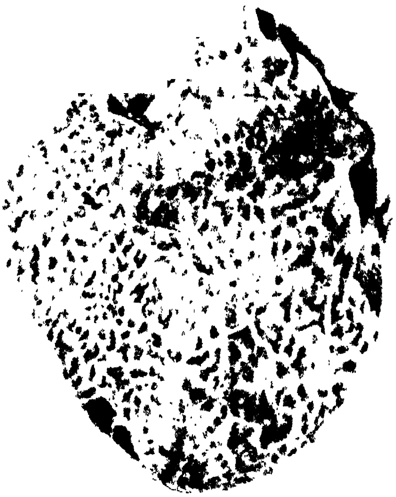


Fig. 147.

Fibrino-purulent pericarditis caused by *D. pneumoniæ*. Note the shaggy appearance of the pericardium.

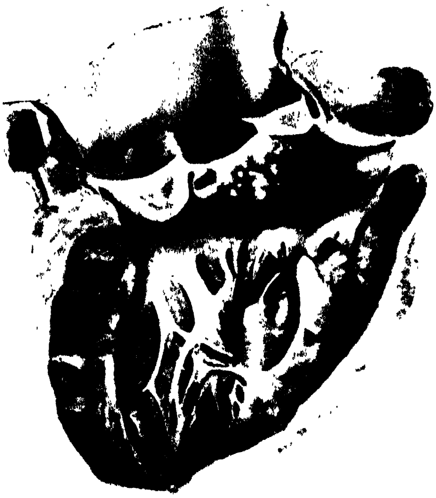


Fig. 148.

Pneumococcal endocarditis of the aortic valve

Note the luxuriant character of the vegetation. There is an opening at one part of the affected valve, showing the friable nature of the vegetation

Endocarditis (ulcerative):—The vegetations are profuse and luxuriant. They are friable and easily break down, giving rise to emboli. The valves affected are usually the aortic (Fig. 148) and the mitral.

NOTE:—Both in pericarditis and endocarditis, the myocardium is always involved. Apart from such cardiac lesions, varying grades of degenerative changes are often found in the heart muscle in pneumococcal toxæmia.

Empyema:—The whole or a part of the pleural cavity may be filled up with a greenish grey purulent fluid mixed with fibrinous flakes. The pleural membranes are considerably thickened and covered with irregular, shaggy-looking fibrin. The empyema may be localised, being completely shut off from the rest of the pleural cavity or the fluid may accumulate in between two lobes forming an interlobar empyema. Two varieties of empyema are described. The one, *synpneumonic empyema*, which occurs in association with pneumonia, often found in children and results in a fatal issue. The other, *metapneumonic empyema*, which occurs after pneumonia is over, is not so serious as the previous type.

Peritonitis:—Primary pneumococcal peritonitis generally occurs in female children, the infection reaching the peritoneum via the Fallopian tubes. In some cases, it may be a manifestation of general septicæmia without lobar pneumonia. But the majority of the cases occurs secondary to some pneumococcal infection in some other parts of the body, such as pneumonia, empyema, otitis media, arthritis or localised abscess. A remarkable feature of this condition is its comparative favourable prognosis. It has been suggested that peritonitis, arising as a secondary complication in lobar pneumonia, may be due to an extension of the infection through the diaphragm, but it is difficult to follow. We are, however, particularly in favour of a hæmatogenous infection.

Acute arthritis (purulent):—This may arise as a complication of pneumococcal infections, the organisms reaching the joint via the blood stream. The organisms can be isolated from the pus aspirated from the joint.

Pyæmia:—Formation of multiple abscesses may, sometimes, be seen during convalescence or towards recovery from the disease. From such abscesses, D. pneumoniæ can be isolated.

Septicæmia: This may arise, either as a primary condition or as secondary to disease in the lungs. In primary septicæmia, the organisms are usually obtained from the naso-pharynx or from minor lesions in some part of the body, such as otitis media. They not only circulate in the blood but, sooner or later, localise in different parts of the body, such as the lungs, meninges and the serous membranes, producing characteristic lesions.

When septicæmia develops as a secondary condition, it is usually the result of a pneumococcal lesion in the body, such as lobar pneumonia. Owing to the diminution of tissue resistance, as well as to the marked invasiveness and toxigenic properties of the organisms, the latter are able to gain entrance into the circulation in large numbers and multiply rapidly, producing severe bacteriæmia and septicæmia. Secondary localisation in the meninges and other serous membranes may also take place as in the primary condition.

Crisis in pneumonia

It has been observed that materials, obtained from the consolidated lung (by lung puncture) from cases progressing towards recovery, show very few Diplococcus pneumoniæ, and that the latter are either degenerated or phagocytosed by the leucocytes. On the other hand, similar examinations in fatal cases reveal a large number of intact D. pneumoniæ, lying free in the exudate. The various factors concerned in bringing about the death and degeneration of the causative organisms in cases terminating by crisis are as follows:

(1) As mentioned before (*vide page 244*), during the growth of D. pneumoniæ, certain substances, such as lactic acid and hydrogen peroxide, are liberated, inhibiting the activities of the organisms. These metabolic products accumulate in large amounts and when a sufficient concentration has been reached, the organisms are rapidly killed.

(2) The specific antibodies (bacteriotropins) are developed in the blood, as a result of the absorption of the specific soluble substance which constitutes one of the toxic products liberated by the organism. Such antibody-formation, of necessity, takes time which is usually about 5 days. Although it has no direct bactericidal effects, it helps phagocytosis by neutralising the specific soluble toxic substance on the surface of the bacteria.

(3) The effects of the extracts of leucocytes and platelets (*leukins and plakins, vide p. 114*) are also responsible, to a certain extent, for the death of the organisms.

THERAPEUTIC CONSIDERATION

Serum therapy:—Specific antisera are available for Type I and also for Type II D. pneumoniæ, but they do not produce any effect in Type III and Type IV pneumonia cases. These sera may be of two kinds, monovalent and polyvalent. The monovalent sera are strictly type-specific and therefore their efficacy will depend on the presence in the antisera of the antibodies, corresponding to the polysaccharide antigens which determine the type specificity of D. pneumoniæ.

Felton has been able to obtain a concentrated polyvalent serum (2,000 units in 1 c.c.) which can be used in the treatment of pneumonia caused by Types I and II D. pneumoniæ. Many observers have reported promising results from the use of Felton's polyvalent serum.

Dosage and administration—The principle that should be adopted in the serum therapy is its early administration in adequate dosage by the intramuscular or preferably, by the intravenous route. It is advisable to use a polyvalent serum in the beginning, as the typing involves a certain amount of delay. When the type of the infecting organisms has been determined, the polyvalent serum can be substituted by the monovalent one. If it is found to be Type III or IV, the injections should be stopped. During the first twenty-four hours, 40,000 to 50,000 units should be administered in moderate cases, and 80,000 to 100,000 units in severe cases. So long as there is bacteriæmia and a high temperature, the injections should be continued every 8 to 12 hours.

Mode of action of antiserum—These antisera are not antitoxic but are chiefly antibacterial in nature, containing immune opsonins *(bacteriotropins)*, *agglutinins* and *complement fixing bodies*. They do not, however, possess any direct bactericidal action but produce lysis of the organisms by promoting phagocytosis and their consequent digestion inside the leucocytes (*vide p.* 246). It should be remembered in this connection that antiserum of one type has no action on infection with other types, except the non-specific effect of serum proteins.

The injections of specific antiserum, specially in Type I pneumonia cases, cause a marked improvement in the general condition of the patient and relieve toxæmia. This specific therapy, however, has no effect on the rate of resolution of the lung tissue already involved, but the lesions cease to extend. It produces a marked effect on bacteriæmia, the blood, where D. pneumoniæ were previously found, becoming sterile.

Normal death rate from pneumonia due to Type I and II, is 25 to 30 per cent., but early administration of adequate doses of specific antisera reduces the mortality to one third.

Vaccine therapy:—The use of pneumococcal vaccines for curative purposes has not been attended with much success. Though the vaccine treatment appears to be valueless in pneumonia, it may be useful in local infections. In order to increase the resistance of the body against the infection with D. pneumoniæ, prophylactic inoculation may be resorted to. For this purpose, three injections are given at weekly intervals, each dose consisting of 6,000 million cocci of each group against which immunity is desired. Such vaccines are often combined with the organisms of common cold. The immunity developed is very short-lived, lasting for a period of about 6 months; owing to this drawback, it is not possible [illegible] stress on general vaccination.

Pneumococcus immunogen combined:—These antigenic substances are derived from the ectoplasm, rather than the endoplasm, of the bacterial cells; hence, it is technically designated as "ecto-antigen." Pneumococcus immunogen combined, contains in each c.c. the antigens from Diplococcus pneumoniæ (3 types), 2,000 millions and Streptococcus (hæmolytic and non-hæmolytic), 2,000 millions. The injections may be given subcutaneously or intramuscularly and are usually given daily. Subcutaneous injections should usually start with 0·1 to 0·2 c.c. It is used usually for curative purposes.

ISOLATION OF DIPLOCOCCUS PNEUMONIÆ IN PURE CULTURE

D. pneumoniæ can be isolated from sputum in lobar pneumonia, blood in cases of septicæmia, cerebrospinal fluid in pneumococcal meningitis and from other pneumococcal exudates, such as pus in empyema, otitis media, corneal ulcers, arthritis, etc. The organism may be isolated in pure culture either on blood agar plate or by animal inoculation method.

1. ***On blood agar plate:*** – After incubation for 24 hours, the colonies of D. pneumoniæ are easily differentiated from all but those of Streptococci. With practice, however, they may be distinguished from the latter by their (a) smooth edges, (b) greater transparency and (c) more flatness. The differentiation between the two organisms is shown in the following table:—

DIFFERENTIATION OF D. PNEUMONIÆ FROM STREPTOCOCCUS

	D. pneumoniæ.	*Streptococcus.*
Morphology:		
Shape .	Lancet or elongated	Typical round or ovoid forms.
Arrangement ..	In pairs.	Usually in chains.
Capsule .	Well defined.	Usually none.
Biochemical Activities: –		
Inulin fermentation ...	+	–
Bile solubility .	+	–
Power of haemolysis ... (Tested on blood agar).	α-haemolysis.	Varies.
Methaemoglobin formation (Tested on blood agar).	Well marked.	Varies.
Serological Reaction: –		
Agglutination reaction with specific antiserum	+	–
Pathogenicity: –		
Virulence for mice ...	Always very high	Variable.

+ indicates positive reaction, indicates negative reaction.

2. ***Animal inoculation method:***—This forms the easiest way to isolate D. pneumoniæ from a mixed culture or infected material, such as sputum, pulmonary exudate, etc. The organisms can be recovered in pure culture from the heart's blood, twenty-four hours after their injection into white mice.

METHOD—A small portion of sputum is emulsified in sterile saline and injected into the peritoneal cavity of a mouse or into the marginal ear vein of a rabbit.

RESULTS—The animal may die of septicæmia in about 2 days' time, but it is usually killed as soon as it shows evidences of illness within 5 to 8 hours. Heart's blood or peritoneal exudate (in the case of mice) is then examined and found to teem with D. pneumoniæ and a pure growth may be obtained by culturing the above on a blood agar plate.

A bacterial suspension is then prepared, which may be utilised for the determination of the type by agglutination reaction. Such a bacterial suspension may also be prepared from the peritoneal exudate of mice, if it contains abundant D. pneumoniæ. After opening the peritoneal cavity with aseptic technique, it is washed with 5 c.c. sterile saline. If the exudate contains cells and masses of fibrin, the peritoneal washings are centrifuged at a low speed for 2 minutes to get rid of them. The supernatant cloudy fluid is pipetted off and put into a second tube which is centrifuged at a high speed for 15 minutes to throw down the bacteria. The clear supernatant fluid is discarded and the deposited bacteria are diluted with sterile saline solution so that the suspension has a density of about 1,000 million organisms per cubic centimetre. This emulsion is then used for macroscopic agglutination.

LABORATORY DIAGNOSIS OF PNEUMONIA AND PNEUMOCOCCAL INFECTION

The diagnosis of pneumonia is the province of the clinician and cannot be placed on the bacteriologist. The latter helps to determine the type of the infecting organism, so as to facilitate the specific serum therapy. The various methods that comprise the laboratory diagnosis include the isolation of the organism and determination of its type (direct methods) and certain accessory (indirect) methods, such as leucocytic response and demonstration of the specific soluble substance eliminated in the sputum and urine.

I. ***Direct method:***—This consists in finding out the causative organism and its type. The materials which are subjected to examinations are sputum or fluid from lung puncture in lobar pneumonia, blood in cases of speticæmia, cerebro-spinal fluid in miningitis or any other pneumococcal exudate. The following comprise the various methods which are adopted in the bacteriological diagnosis.

I. **Microscopical examination** A film, prepared from the suspected material, when stained by Gram's method, will reveal the *Gram-positive*, characteristic *lance-shaped*, *capsulated diplococci*.

2. **Cultivation**—Culture should be made in blood agar. If D. pneumoniæ is present, it will be recognised by its characteristic colonies. In 25 to 30 per cent. of pneumonia cases, the organism can be isolated from the blood, if 5 to 10 c.c. are taken for culture.

3. **Biochemical reactions**—Bile solubility and inulin fermentation are then tested. These two tests are quite adequate for the identification of the organism.

4. **Animal inoculation**—To test the pathogenicity, a white mouse may be inoculated intraperitoneally with the culture material or infected substance. The occurrence of septicæmia with the characteristic diplococci in the heart's blood or in the peritoneal exudate is a conclusive proof of the identity of the organism.

5. **Type determination or serological identity** can be ascertained by one of the following: agglutination method, precipitin method, or direct method. The material commonly examined for this purpose is sputum. These methods are described later.

II. ***Indirect methods:—***

(1) **Blood count**—A leucocyte count is often done as an aid to diagnosis and a high leucocytosis is in favour of a pneumococcal infection. It should be remembered that there are cases where such leucocytic response is not marked. This is particularly so with Type III pneumonia which invariably ends fatally. Polymorph [illegible]

(2) **Demonstration of the specific soluble substance** in the sputum and urine (*vide infra*)—Owing to excretion of the soluble antigenic substance in urine, a specific precipitin reaction may be obtained to identify the type of the organism. This substance may be demonstrated in the urine of over 60 per cent. of pneumonia cases. It is excreted within the first day after the onset of the disease and may continue to be eliminated in the urine up till early convalescence (Dochez and Avery).

Methods for determining the types of Diplococcus pneumoniæ

Typing of D. pneumoniæ is very important, as it serves an useful guide in the treatment of pneumonia with the specific antiserum. The various methods which are in use fall under three heads:—(1) agglutination method, (2) precipitation method and (3) direct method.

1. **Agglutination method:**—The technique involves (a) the isolation of the organism in pure culture by mouse inoculation (*vide supra*) and (b) testing the agglutination reaction with stock antisera. The reactions may be carried out *macroscopically* and *microscopically*.

A. *MACROSCOPIC AGGLUTINATION:*—This test is carried out in a series of 5 small test tubes and is set up along with the bile-solubility test as follows:—

	For agglutination.			For bile solubility.	For control.*
Tubes (Dreyer's)	1	2	3	4	5
Diluted antisera (stock).	0·5 c.c. of serum I. (1 : 20)	0·5 c.c. of serum II. (1 : 20)	0·5 c.c. of serum III. (1 : 5)	0	0
Sterile ox-bile or 10 per cent. solution of pure sodium taurocholate. ...	0	0	0	0·1 c.c.	0
Bacterial emulsion (D. pneumoniæ).	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.

The tubes are placed in the incubator at 37° C. for one hour.

Reading of the results:—

(a) The bile-solubility is recognised by clearing of the turbidity in tube 4.

(b) The tubes 1, 2 and 3 are examined for agglutination which is read macroscopically, the reaction being indicated by clouding and flocculation. The tube in which agglutination occurs determines the type of D. pneumoniæ under consideration.

(c) Strains soluble in bile, but not agglutinated by any of the 3 type sera, are placed in Group IV.

Drawbacks of this test are the use of animals and the delay which takes place in obtaining a pure culture of the organisms.

B. *MICROSCOPIC AGGLUTINATION TEST:* Without waiting for the death of the mouse, a small amount of the peritoneal fluid may be withdrawn by puncture, within 3 to 6 hours after the injection of the sputum. A glass slide is marked off into four parts, and a minute drop of the peritoneal fluid is expelled on each of the four partitions. The first is smeared with saline for control, and the others with a loopful of 1 : 10 dilutions of type I and II, and a 1 : 5 dilution of type III antisera respectively. These dilutions of the sera are chosen largely to eliminate group agglutinins. The materials are well mixed and uniform thin smears are made. These are allowed to dry rapidly and fixed by heat. They are then stained for 20 to 30 seconds with a dilute fuchsin solution (10 c.c. saturated alcoholic solution of basic fuchsin and 90 c.c. of water) or any other available stain. The stain is washed off in water or 20 per cent. copper sulphate solution, and the smears are examined with the oil immersion lens. A positive agglutination may be ascertained from the aggregation of the cocci into clumps, leaving the intervening spaces fairly free. The type serum which gives rise to agglutination of the organisms will determine the type to which they belong. If no reaction occurs in any of the smears, and numerous D. pneumoniæ are clearly seen, a diagnosis of Group IV is suggested.

This method may also be applied to blood cultures by centrifugalising the growth in broth and using the sediment, and often directly to spinal fluid, provided the organisms are sufficiently numerous.

2. **Precipitation method**:—The principle underlying this method is that D. pneumoniæ produce a soluble substance which gives a specific precipitin reaction with the homologous antipneumococcal serum. Such a substance can be demonstrated in the sputum, inflammatory exudates and urine of patients suffering from lobar pneumonia.

(a) FROM SPUTUM

Technique:—(Oliver's method—time required 30 minutes).

(i) Select the most purulent or blood streaked portion of the sputum. Make a smear and stain by Gram's method to confirm the presence of D. pneumoniæ.

(ii) Place 1 to 2 c.c. of the sputum in a centrifuge tube and add 3 to 5 drops of a 10 per cent. sodium taurocholate solution.

(iii) Thoroughly break up and mix the mass by stirring with a rod, adding just enough normal saline drop by drop.

(iv) Place the tube in a water bath at 42° to 45°C. for 20 minutes.

(v) A small bit of cotton wool, with fibres loosely united, is placed at the top of the fluid in the tube which is centrifugalised at high speed. The cotton is pushed down to the bottom to assist in clearing the solution.

(vi) Carefully pipette off the supernatant fluid and distribute into 3 small absolutely clean test tubes, 0·3 to 0·5 c.c. into each. Label the tubes 1, 2 and 3.

(vii) To these tubes, add 1 to 2 drops of Type I, II and III pneumococcal antiserum respectively. A positive test is indicated by an almost immediate clouding and flocculation which is increased by heating in a water bath at 42°C. for 15 minutes. The tube in which this occurs indicates the type of the organism present in the sputum.

(b) *FROM URINE*:—Precipitation reaction may also be carried out with a sample of urine of a patient suffering from lobar pneumonia. This method is of less value because the sputum examination is applicable earlier and the demonstrable amounts appear in the urine, usually in severe cases of pneumonia. The reaction is set up exactly in the same way as for sputum with a clear centrifuged urine, in which the antigen has been concentrated by boiling with acetic acid and then precipitating with 95 per cent. alcohol.

3. **Direct method:**—Neufeld in 1902 observed that when a suspension of D. pneumoniæ was mixed with homologous immune serum on a slide, not only agglutination, but also a marked swelling of the capsules occurred *(Quellung pnenomenon of Neufeld)*. Armstrong (1932) applied this observation to direct methods of typing of D. pneumoniæ from sputum.

ARMSTRONG'S TECHNIQUE (British Medical Journal, Jan. 30, 1932):—Three small samples from a suitable fleck of sputum are placed, equidistant, on a microscope slide, and numbered 1, 2 and 3. Each sample is emulsified with four times its volume of the corresponding diagnostic serum (antiserum), the addition of the serum and emulsification being conveniently performed with a platinum loop. Cover-glasses are applied and the slide is left under cover for a few minutes, whilst a further sample from the selected fleck of sputum is smeared on a slide, fixed by heat, and stained by Gram's method. The general bacterial flora of the sputum and the number of D. pneumoniæ present are apparent at a glance in the stained film. The slide containing the fresh emulsions of sputum with the specific sera is now examined, using a 4 ocular and 1/6 objective and a plane mirror, the condenser being removed. When the reaction is negative, the unstained D. pneumoniæ are barely visible. In the case of a positive reaction, on the other hand, there is a conspicuous increase in the size of the individual D. pneumoniæ, due to conjugation of the coccus with the homologous antibody. The enlarged cocci have a characteristic ground-glass appearance, with a highly refractile peripheral zone. A positive reaction is at times appreciable to the naked eye on holding the preparation to the light. The positive, as compared with its negative companions on the same slide (which serve as controls), is seen to be opalescent, due to the great increase in the size of the "sensitised" cocci. When the D. pneumoniæ are thickly coated with a sero-mucinous secretion, the characteristic appearances develop more slowly, as the specific serum takes time to soak its way through. In such cases, twenty minutes may elapse before the completion of the test, although type may be distinguished much sooner by the change in those cocci which are floating free. It is to be noted that the type is decided independently of the actual agglutination of the organisms. This is prevented, presumably, by the viscous nature of the sputum.

"*Commentary*:—The results of the "direct test" have been confirmed in every case by mouse inoculation, and by the other more deliberate methods commonly used in the labo atory. The direct method has proved trustworthy, and its value lies in extreme simplicity and swiftness. The objection now disappears that the pneumococcal type cannot be decided in general practice, since animal inoculation is no longer essential and a special knowledge of the bacteriological technique is not necessary. Type may be decided with equal ease and speed, by the direct method, in the case of cerebrospinal fluid, pus from empyemata and aural discharges."

CHAPTER XXI

NEISSERIA GONORRHŒÆ

(Gonococcus; Micrococcus gonorrhœæ)

HISTORICAL: *Albert Neisser* (1879) first gave an account of the microscopic characters of this organism as seen in the pus of gonorrhœal affections.

HABITAT:—The organisms are never present in normal individuals, but always in the lesions in persons suffering from gonorrhœa. They are readily found in large numbers in the urethral discharge of a patient suffering from acute gonorrhœa.

MORPHOLOGY:—

Size: The organism is a micrococcus, each being about 1 μ in diameter.

Shape:—It is reniform in shape, often compared to a bean or a kidney.

Arrangement:—These cocci are arranged in pairs (hence called diplococci), with their concave margins apposed to each other, so that between there is a small oval unstained space.

Involution forms are often seen in old cultures. These are cocci in the process of degeneration, appearing as spherical elements of varying sizes, some being considerably swollen and staining faintly.

The organisms are non-capsulated, non-sporing, non-flagellated and non-motile.

These diplococci are usually found in large numbers within the cytoplasm of the polymorphonuclear cells and are often so numerous as to obscure their nuclei. This intracellular position of the cocci constitutes one of their special characteristics (Fig. 149).

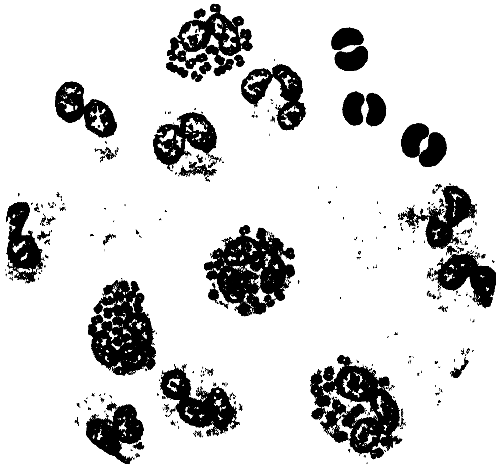


Fig. 149.

Neisseria gonorrhœæ in a smear of urethral pus from a case of acute gonorrhœa. Note the intracellular position of the diplococci. The characteristic reniform appearance (bean shape) of the cocci is shown in the inset.

STAINING REACTIONS:—1. With basic aniline dyes, such as methylene blue, basic fuchsin, etc., the organisms are stained readily.

2. By Gram's method of staining, they are easily decolourised, hence they are Gram-negative.

Note:—*In a smear of urethral pus, the presence of Gram-negative, intracellular, reniform, diplococci is diagnostic of gonorrhœa.*

BIOLOGY:—The organism is an aerobe, but grows best in lowered oxygen tension (micro-aerophilic). It grows best at a temperature of 35° to 36°C., and the growth ceases altogether at 25°C. The optimum pH is from 7·3 to 7·6. The organism requires abundant moisture for its growth. It is extremely sensitive to heat, antiseptics and sunlight.

Fig. 150.

Colonies of *Neisseria gonorrhœæ* in hydrocele agar. Some colonies of Staphylococci are also shown in the upper and left part of the tube.

CULTIVATION:—It is not possible to cultivate the organisms in ordinary media, such as agar or broth. They grow best when some uncoagulated body protein, such as blood or serum, is incorporated in the culture medium. The best medium, therefore, is agar containing serum, whole blood, ascitic fluid or hydrocele fluid. Culture of the infective material should be made direct from the discharge on to the medium and incubated without delay. The colonies are visible within 24 to 48 hours. Sometimes, the growth may not appear until the fourth day.

Note:—Heated blood agar forms an excellent medium for the cultivation of N. gonorrhœæ. By heating the blood to 55°C. certain amino-acids, having growth-inhibiting properties, are eliminated.

Character of the colonies:—These tend to remain more or less discrete. They appear as semi-transparent discs, about the size of a pin's head. At first the margin is rounded, but later it becomes undulated or crenated and the centre also becomes more opaque (Fig. 150).

The organisms die out in the primary culture very soon. Hence, subcultures should be made within 2 to 3 days. In subcultures, however, which are kept at 37°C., the organisms remain alive for a considerable time, sometimes as long as 3 weeks.

BIOCHEMICAL REACTIONS:—

Fermentation reactions—The fermentation of the two sugars, glucose and maltose, is very important and is utilised for the differentiation of the Gram-negative diplococci, as shown in the table on page 265.

Table showing differences in the fermentative activities of the Gram-negative cocci.

Organism.	Glucose.	Maltose.
N. gonorrhœæ ...	+	o
N. meningitidis ...	+	+
N. catarrhalis ...	o	o

+ = Fermentation; o = No fermentation.

Neisseria gonorrhœæ ferments glucose only, with the production of acid but no gas. It does not ferment maltose. The fermentation tests are best demonstrated on solid media in which brom-cresol purple may be used as the indicator. The preparation of such a solid medium may be carried out by adding 1 part of a 10 per cent. solution of the requisite sugar along with the indicator to 10 parts of melted nutrient agar at 55°C. The mixture may be sterilised by the fractional method.

VIABILITY:—Neisseria gonorrhœæ is a very delicate organism and it undergoes autolysis readily in culture. It can not withstand heat, desiccation, sunlight or antiseptics to any appreciable extent. In cultures exposed to 40°C. for a few hours, the organisms usually die. A temperature of 107°F. (42°C.) kills the organism in the infected tissues in about an hour. This fact should be remembered in connection with the treatment of gonorrhœal affections by means of diathermy. The organisms are also very susceptible to the action of silver salts, such as argyrol, protargol and others, in concentrations which do not harm the mucous membrane of the urethra.

TOXIN PRODUCTION:—Exotoxins have never been satisfactorily demonstrated. Endotoxins are responsible for the various effects which are produced by the N. gonorrhœæ. Inoculation of the human urethra with this endotoxin causes an acute catarrh. It should be noted that N. gonorrhœæ undergoes autolysis very soon in culture; hence, the filtrate of a fluid culture of a few days' old, will contain the endotoxins.

SEROLOGICAL REACTIONS:—Specific antibodies, such as (i) agglutinins and (ii) complement-fixing immune bodies, have been demonstrated in the serum of persons infected with N. gonorrhœæ. Hence, agglutination reaction and complement fixation test may be utilised as accessory aids in the diagnosis of a gonococcal infection. As such sera do not possess any bactericidal or antitoxic property, they cannot be used therapeutically.

ANIMAL INOCULATION:—It is virulent only to human subjects. No animals are ever affected; even intraurethral inoculation in apes failing to show any disease. In fact, it has been found impossible to reproduce the disease by any means in the lower animals. The autolysed bacterial protein, on the other hand, is highly toxic to labor[illegible] animals.

PATHOGENICITY

Neisseria gonorrhœæ produces the disease gonorrhœa in man, which is characterised by a specific contagious inflammatory discharge from the mucous membrane of the urethra or vagina. The word gonorrhœa literally means a discharge or flow from organs related to generation (*gonos*, generation or seed; *rheein*, flow). *It is exclusively a human disease and animals do not contract gonorrhœa.* The organism is primarily infectious for the genital organs and the infection is almost always direct, from person to person, and usually results from sexual intercourse. Mechanical infection may take place in some cases, particularly in conjunctival infection and vulvo-vaginitis of young female children.

Portals of entry:—These are two in number, (i) genito-urinary and (ii) conjunctival mucous membrane.

(i) *Genito-urinary mucosa*—This is, by far, the commonest route and the majority of cases occur through infection by this channel.

(ii) *Conjunctival mucosa*—Only local infection is produced and general manifestations are not common.

It should be remembered in this connection, that Neisseria gonorrhœæ shows a predilection, in every case, for **structures lined with columnar epithelium,** because the stratified and squamous epithelia offer a greater resistance to the entrance of this organism. Hence, the vaginal mucous membrane in adult females and the penile portion of the urethra in males, both of which are lined with stratified epithelium, resist infection by these cocci. In adult females, vulva and vagina are not liable to be attacked until the squamous epithelium covering them becomes sodden and devitalised by discharges from some other parts of the genito-urinary tract. Such a condition is present during pregnancy and puerperium, when the vaginal mucous surface becomes much more prone to gonococcal infection. A purulent form of gonococcal vulvo-vaginitis, seen in young girls, may be explained by the fact that upto puberty the vulva and vagina are covered with cylindrical epithelium which favours the infection.

Pathology:—The organisms quickly penetrate the mucous membrane, passing chiefly between the epithelial cells which are loosened and desquamated, and finally invade the submucous tissues. After *an incubation period of 2 to 10 days*, an acute catarrhal inflammatory reaction ensues, producing congestion, œdema, and an extensive leucocytic emigration. This is associated with much increase of secretions. The discharge is at first mucoid, but later becomes purulent. The acute stage may pass off in a few days and, under favourable conditions, may undergo complete resolution. But even at an early stage, the Neisseria gonorrhœæ may penetrate into distant parts by the lymphatics. Thus, in a large number of cases, a subacute or chronic inflammatory condition is maintained by the organisms which get into the crypts and recesses of the urethral and genital passages. From these places they are capable of keeping up the infection.

In the earliest stages, when the discharge is glairy, the organisms may be found lying free in the exudate, but later, when it is purulent, they are found packed within the leucocytes. Phagocytosis of the organisms takes place in the exudate and not in the inflamed tissue. Within a few weeks, Neisseria gonorrhœæ become scanty in the discharge and are replaced by secondary organisms, such as Streptococci, Staphylococus pyogenes albus, Escherichia coli, Diphtheroid bacilli and others. During the more chronic stages, these organisms aid in maintaining the irritation and may be responsible for some of the secondary results.

The rôle of secondary infections in gonorrhœa:—The secondary organisms, as mentioned above, are present in chronic and badly treated cases and are most often found in females. These bacteria undoubtedly play some part in maintaining a subacute or chronic inflammatory process in the urethra and its diverticula. Moreover, they cause difficulty in the bacteriological diagnosis of the condition. Because, the Gram-positive cocci of secondary infection may, specially when phagocytosed, lose their Gram-positive character, and are then liable to be mistaken for intracellular Gram-negative cocci. Then, again, the examining eye is liable to be diverted and deceived by the presence of a large number of secondary organisms.

Lesions: The various lesions produced by Neisseria gonorrhœæ may be divided into 2 main groups, (1) primary and (2) secondary.

Primary lesions: These are tabulated as follows:—

A. *In males*—Anterior part of the urethra is first affected, resulting in an acute catarrhal inflammation which proceeds later on to pus formation (anterior urethritis).

B. *In females*—The urethra is the most common starting point, the situation affected next in frequency being the cervix uteri (urethritis and endocervicitis).

C. *In children*—Gonorrhœa occurs as an anterior urethritis in males and vulvo-vaginitis in females. This is in most cases due to the carelessness of the nurse or mother carrying the infection.

D. *In the new-born infant*—A frequent accidental infection at birth is gonorrhœal ophthalmia (purulent conjunctivitis).

Secondary lesions:—These are produced by the following ways:—

(i) By direct extension.

(ii) By infection of the lymph stream.

(iii) By infection of the blood stream.

(iv) By mechanical means.

(i) *RESULT OF DIRECT EXTENSION*—In this case, the infection spreads through continuity and contiguity of mucous membranes. A knowledge of anatomy is essential to understand these lesions.

In males (Fig. 151)

There are, in the course of the urethra, many accessory structures, such as (i) paraurethral ducts and subpreputial sac, (ii) Littré's glands and their ducts, (iii) Cowper's glands and their ducts, (iv) prostate gland and its ducts, (v) common ejaculatory ducts, seminal vesicles, vas (ductus) deferens and the epididymes and (vi) prostatic utricle. All of these may be involved in the more chronic gonorrhœal infections, inspite of a thorough disinfection of the urethra itself. Moreover, in many cases, an extension into the posterior urethra takes place where healing is more difficult and from which important complications in other organs may arise. Certain important sequelæ which may result in the course of a gonorrhœal infection are urethral stricture and chronic prostatitis and vesiculitis.

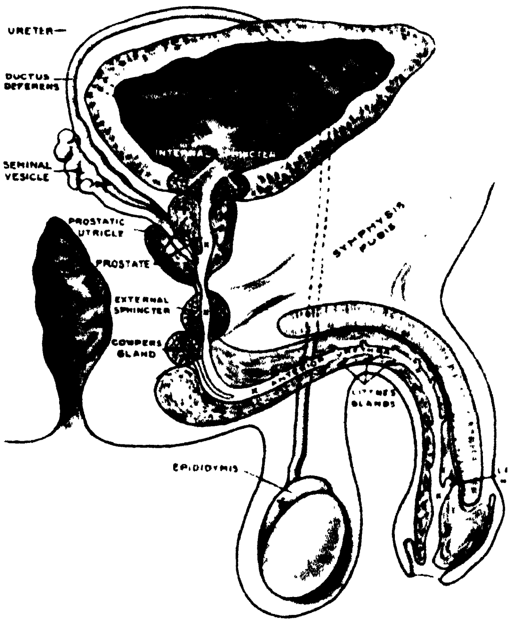


Fig. 151.

Anatomy of the male genito-
various sites of the lesions a

1. *Urethral stricture*.—The commonest site for this condition is the junction of the bulbous and membranous urethra, as this is the most dependent part of the urethral tract and is therefore the site at which purulent discharge tends to accumulate, if good drainage is not established during treatment. The presence of the sphincter muscle in this area also determines very greatly the occurrence of stricture. During contraction of the sphincter, whose action is exaggerated if the urethra is sensitive, the inflamed and ulcerated passage is constantly subjected to injury, causing fissures and rupture of the granulations, resulting in the formation of an excess of cicatrix and consequent stricture.

2. *Chronic prostatitis and vesiculitis*—These serve as reservoirs of infection and, owing to their high vascularity, on many occasions, are responsible for serious metastatic complications.

In females

The urethra of the female being shorter and without any complicated accessory structures, the inflammation does not lead to such serious results as are found in males. But the internal genitalia are particularly involved, due to an extension from a gonococcal endocervicitis. The following are the common lesions met with in women (Fig. 152):—*Bartholinitis and Bartholin abscess; endometritis; salpingitis and oöphoritis, pyosalpinx, tubo-ovarian abscess; pelvic peritonitis.*

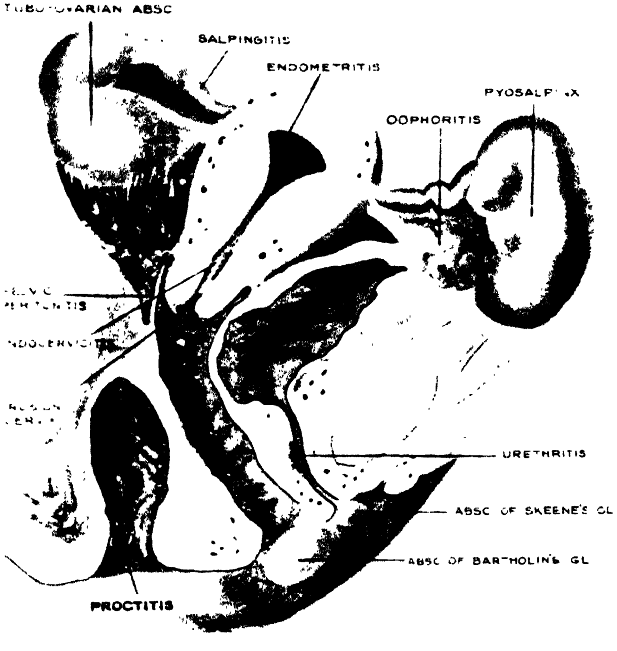


Fig. 152.
Gonorrhœal lesions in women (Diagrammatic)

It should be remembered that the mucous membrane of the anus and rectum, on rare occasions, may be involved in both sexes, though the lesion is particularly common in the female sex.

(ii) *INFECTION BY THE LYMPH STREAM*:—The complications that commonly arise by this method are epididymitis (in male), cystitis, pyelitis and pyelonephritis. These are generally produced by secondary invaders. This method of infection is often referred to as the "*ascending infection*" of the genito-urinary tract, the infection travelling up towards the kidneys. This is possibly due to the free inter-communication between the lymphatics of the genito-urinary tracts.

The above infection, under certain circumstances, may spread by direct continuity through the lumen. It happens when there is some obstruction in the urinary tract producing a back pressure. Lastly, the above complications may also arise from a hæmatogenous infection.

In this connection, it should be noted that the common *bubo* is the direct extension of infection through the lymphatics.

(iii) *BY INFECTION OF THE BLOOD STREAM:*—It has already been mentioned that this is produced, on many occasions, as a result of the presence of some focus of the disease in the genito-urinary tracts of either sex. In the male, the original focus of the disease is found most frequently in the posterior urethra, specially in the prostate gland, seminal vesicles and the epididymes. In the female, the focus of the disease is commonly in the endocervix and Fallopian tubes. General blood infection is particularly common in males.

After once the organisms get into the blood stream, they may produce a septicæmia or may localise in the heart, joints and eyes, giving rise to various metastatic lesions. In the *heart*, the organism involves the valvular endocardium and the nature of the lesion resembles that of the other pyogenic cocci. The vegetations which form are luxuriant, easily friable and break down. Such ulcerative endocarditis is found in association with septicæmia and invariably ends fatally.

Gonorrhœal arthritis (Gonorrhœal rheumatism) They are met with in all stages of gonorrhœa, either acute, subacute or chronic. The synovial membranes of the joint, tendon sheaths, ligaments and fibrous aponeuroses are commonly involved. The arthritis may be monoarticular as well as polyarticular and certain joints are more commonly involved than others, such as temporo-mandibular, sterno-clavicular and vertebral joints. The affected joint becomes swollen as a result of collection of fluid inside the synovial membrane. The organisms may be isolated from the fluid aspirated from such a joint.

Gonorrhœal iritis—Apart from conjunctivitis, iritis is another ocular complication met with in gonorrhœa. This is one of the manifestations of metastatic lesions, and, under certain circumstances, the whole of the uveal tract may be involved. Isolation of the organism from such lesions is very difficult owing to its inaccessible position.

(iv) *BY MECHANICAL INFECTION:*—

Conjunctivitis—A purulent form of conjunctivitis may be produced as a result of direct inoculation, being generally transferred by fingers or by some infected towels, handkerchiefs and clothings. Thus, in a person suffering from an acute discharge, there is always the danger of self infection. Hence, the necessity of personal cleanliness is very important to prevent such spread. Other persons who are liable to such infection are physicians and nurses.

Relation of Gonococcal infection to Pregnancy and Conception

This may be considered under 3 headings:—

1. **Ophthalmia neonatorum**—If the infection is present in some part of the birth canal, the organisms may gain entrance into the eyes of the baby

during its passage and produce a very bad inflammation of the conjunctiva. This may ultimately lead to permanent blindness. In any suspected case, silver salts (1 to 2 per cent. silver nitrate solution) should therefore be instilled into the eyes of the new-born baby as a prophylactic measure. After allowing the solution to remain for a few seconds, it may be washed off with normal saline *(Credé's method)*.

2. **Sterility**—The question of sterility in gonococcal infection is a very serious one. The danger is more in the female than in the male. The spread of the infection to the tubes and ovaries and the resulting inflammation in these organs, specially when bilateral, preclude all possibilities of conception. Similarly, when seminal vesicles, epididymes and the testes are affected in the males, the risk of sterility is potentially present.

3. **Abortion**—Owing to the production of a chronic unhealthy condition of the endometrium, the implantation of the ovum and its subsequent development are interfered with, particularly in the early months of pregnancy. This may give rise to repeated abortions.

If, however, the infection is contracted during pregnancy, when the ovum has completely filled up the uterine cavity, the usual course of gestation is hardly interfered with. Hence, there would be no symptoms as far as the uterus is concerned, but there may be free leucorrhœa (white discharge) from the pathological condition in the cervix as well as in the urethra.

THERAPEUTIC CONSIDERATION

Serum therapy: Specific antisera in the treatment of gonorrhœa are practically not useful at all.

Vaccine therapy:—This may be of some value in joint affections and allied conditions, but there is no proof of its value in an ordinary case of specific urethritis. The initial dose of the vaccine is generally 5 millions. The success which is sometimes obtained from the therapeutic use of gonococcal vaccines may be due not to its specific nature, but to the reaction following the use of a non-specific protein. Hence, treatment by gonococcal vaccines has now-a-days been largely replaced by non-specific protein therapy. As the plain gonococcal vaccine is very toxic and gives rise to untoward effects, various preparations have been introduced for this purpose. These are sensitised vaccines, detoxicated vaccines and phylacogens. By using such preparations, large doses may be used without their corresponding toxic effects.

In very chronic cases, where secondary infection is very marked, it is advisable to use a mixed vaccine.

LABORATORY DIAGNOSIS

The diagnosis of gonorrhœa is very important, not only in clinical, but also in medicolegal, cases.

Collection of materials:—During the earlier stages of the disease, it is not very difficult to obtain the material and the diagnosis can easily be made from the purulent exudate by finding the characteristic Gram-negative, intracellular diplococci. It is the chronic cases which give rise to difficulty, because the bacterial flora have changed and the place of the Neisseria gonorrhœæ has been taken up by various secondary organisms. Besides, the causative organisms have taken shelter in the deeper tissues, viz., in the crypts and recesses, which are difficult to reach. Thus, in order to diagnose chronic gonorrhœa (gleet) and to obtain material for the purpose, one requires an intimate knowledge of their lurking places. These are all the anatomical orifices and crypts in connection with the genito-urinary tracts. The most important ones are the seminal vesicles and the prostate gland in the male, and the cervix uteri, urethra and Bartholin's glands in the female. Thus, in males, the materials are better obtained after massaging the prostate gland and the seminal vesicles. In females, a cervical swab or a urethral secretion, obtained by milking the urethra, will help in establishing a bacteriological diagnosis. In this connection, it should be remembered that in females, specimens taken just after a menstrual period are more likely to yield positive results than those taken at normal times. A vaginal smear is useless, as the soil is not suitable for the development of N. gonorrhœæ, except in young children.

Prostatic massage--Patient undergoing prostatic massage should be instructed not to pass urine 2 to 3 hours previously. Just before the procedure, he is asked to pass a small amount of urine (3 to 4 oz.) and the rest is allowed to remain in the bladder. The finger protected with a glove is passed into the rectum with the patient standing but bent over (Fig. 153). Pressure is made on the prostate gland with finger strokes having a movement downwards and medialwards, as shown in Figure 154. The drops of discharge expressed by the massage are then received in a small sterile Petri dish. Some portion of the material which is left in the urethra is finally passed along with the remaining portion of the urine from which a deposit is obtained by centrifugalisation. It should be remembered that prostatic massage is not only done for diagonstic purposes, but is also employed as a therapeutic measure. When used for the latter purpose, it acts in two ways:--(i) by mechanically emptying the prostate gland and seminal vesicles of their pathological contents, and (ii) by improving the circulation of the organs, it helps the process of repair.

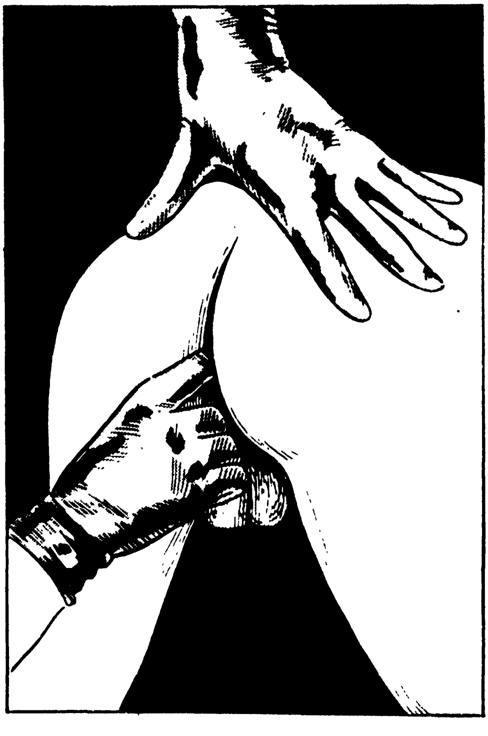


Fig. 153.

Position of the patient during prostatic massage.

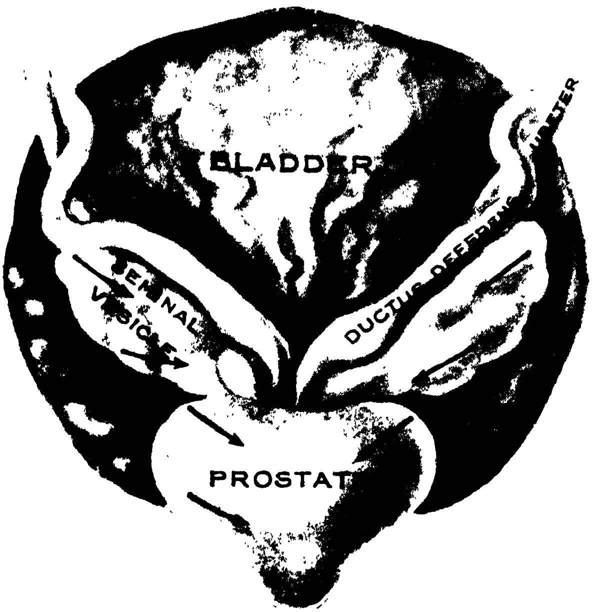


Fig. 154.

Method of doing prostatic massage. The arrow indicates the direction of finger strokes (after Gray's anatomy).

Cervical swab In obtaining a specimen from the cervix uteri, a Fergusson's speculum is passed into the vagina and the external os exposed, excess of mucus or mucopus being removed by mopping with a sterile cotton wool. A suitable sterile swab is then passed into the cervical canal and well rubbed on the mucous membrane.

Urethral secretion in females –This is obtained by running the finger along the anterior vaginal wall from within outwards, so as to express the fluid from the meatus (milking the urethra). In order to obtain good material from the urethra, the patient should be asked not to pass urine for at least two hours before submitting to examination.

Methods of Diagnosis:—

I. ***Direct method:***—This consists in the establishment of a bacteriological diagnosis by (i) microscopical and (ii) cultural examinations.

For *microscopical examination*, Gram's method of staining is the only reliable one for diagnostic purposes. The presence, in the urethral discharge or in any suspected material, of a large number of diplococci, having the characteristic morphology and staining reactions (*Gram-negative intra-cellular*), is practically conclusive of the case being one of gonococcal infection.

In cases where microscopic examination alone is not sufficient for the diagnosis, *cultural methods* should be resorted to, when the N. gonorrhœæ may be identified by its various cultural characters and fermentation reactions which have been already described.

II. ***Indirect method:—***

(i) **Two glass test:**—This is very helpful in deciding whether the posterior urethra is involved or not. Collect the first half of the urine in one glass and the second half in another. If the latter sample remains turbid, after the addition of a few drops of acetic acid to dissolve the phosphates, the posterior urethra is most probably involved.

Prostatic threads—These consist of mucus with entangled leucocytes and epithelial cells and are mostly voided with the first portion of the urine. They are much larger than tube casts, being visible readily enough with the naked eye, as they float in the urine. Their presence always signifies the existence of a chronic inflammation in the posterior urethra.

(ii) **Serum diagnosis:—**

(a) *Complement fixation test:*—This test is sometimes performed for the diagnosis of gonorrhœal infections in which the organisms cannot be demonstrated by direct methods. The test is not very reliable and its interpretation is a matter of difficulty. Early and acute cases rarely exhibit a positive reaction. The test is of considerable value in diagnosing cases of iritis, arthritis and epididymitis that appear as late manifestations. On account of the various types of N. gonorrhœæ, it is essential that the antigen should be polyvalent, consisting of a large number of strains sufficient to represent the different serological races of the organism.

(b) *Agglutination reactions:* This is found only in cases of some duration. The blood serum of the infected person may show a positive agglutination reaction with an emulsion of N. gonorrhœæ. The rôle of agglutination reaction in the diagnosis of gonococcal infection is, however, not very important.

(iii) **Provocative methods in diagnosis:**—The principle underlying this test is to stir up any latent infection in an apparently cured patient and provoke a discharge. The material obtained from the provocative methods is frequently found to be positive in patients who have been free from discharge for some time. Thus, by this method, the organisms can be demonstrated in chronic cases where it is extremely difficult to find them. The methods fall under two groups:—

(a) *Specific*—This consists in an injection of a large dose of gonococcal vaccine (500 millions). Reactions, such as focal (at the site of infection), local (at the site of inoculation) and general (malaise, fever, etc.), occur in an infected person. If no focal reaction occurs, it is improbable that there is any latent focus.

(b) *Non-specific*—(1) Local application of *silver salts*, which leads to irritation and flow of discharge, thus bringing the organisms to the surface from a deep seated latent infection.

(2) Drinking a large quantity of *alcohol* often produces congestion of the parts and provokes a discharge. This is often very helpful.

CHAPTER XXII

NEISSERIA MENINGITIDIS

(Meningococcus)

HISTORICAL:—Weichselbaum, in 1887, first described this organism and established its causal relationship with epidemic cerebrospinal meningitis. He also named the organism as *Diplococcus intracellularis meningitidis.* This name describes the characters of the organism, such as its tendency to infect the meninges, its occurrence in pairs and its position inside the polymorphonuclear leucocytes. It is frequently termed the *Meningococcus.* Its new nomenclature is *Neisseria meningitidis.*

HABITAT:—Primary habitat of N. meningitidis, both in actual "cases" of the disease and in "carriers," is the throat and the upper part of the nasopharynx. Thus, the organism is strictly a *parasite,* of man only, being mostly confined to the nasopharyngeal mucous membrane where it excites a mild catarrhal inflammatory reaction, resulting in a copious mucopurulent or viscid discharge. *Pathologically,* it is found in the exudates on the meninges and in the cerebrospinal fluid. In fulminating septicæmic cases, it can be recovered from the blood.

MORPHOLOGY:—

Size:—It is a small coccus, measuring about 0·8 μ in diameter.

Shape: The separate cocci are plano-convex.

Arrangement:—It usually occurs in pairs, with the flattened surfaces apposed to each other. Although the diplococci are the commonest forms, single coccus or small groups, particularly in tetrads, are also seen. In most cases, the cocci are chiefly contained within the pus cells (intracellular), but they are not so numerous as in the case of a gonococcal infection. The intracellular position is not a site of predilection but is related to the degree of virulence of the strain involved, and the cocci may be seen both intra, and extra, cellular, those of low virulence being readily taken up by phagocytes.

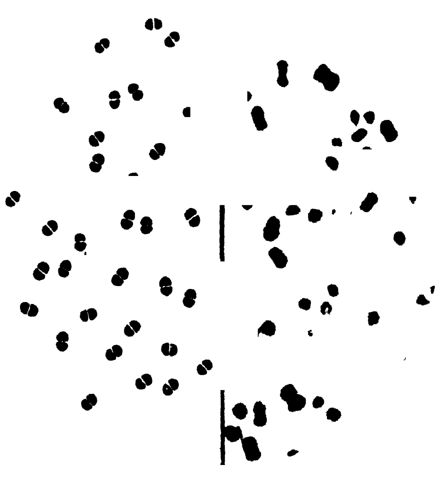


Fig. 155.

The normal (A) and involution (B) forms of *Neisseria meningitidis.*

The organism is non-spore-forming, non-motile and does not possess any capsule.

Involution forms (Fig. 155):—There is a great tendency for these organisms to show involution forms, the cocci losing their normal shape, size and staining characters. They become round, irregular and much swollen, staining badly (*ghost forms*) and afterwards undergoing disintegration. Such *ghost* forms can chiefly be seen in cultures and, also to a less degree, in the inflammatory exudates. Flexner observed that the production of an autolytic enzyme induces these degenerative changes in the cocci.

STAINING REACTIONS:—It stains readily with aniline dyes and when stained by Gram's method, it becomes Gram-negative. As a counter-stain, 1:20 dilution of Ziehl-Neelsen's carbol fuchsin is found to be most satisfactory.

CULTURAL CHARACTERS:—

1. The organism is an obligatory aerobe. The growth of N. meningitidis is definitely stimulated by replacing about 10 per cent. of the air by CO_2 gas.

2. Growth takes place best at the body temperature (37°C.) and it does not usually occur at a temperature below 23°C.

3. The optimum reaction of the medium required is pH 7·4.

4. No growth occurs in ordinary agar or broth. The organism requires blood or serum or any other native albuminous fluid for its cultivation. Hence, the following media are employed for this purpose, (i) *blood agar*, (ii) *serum agar* and (iii) *hydrocele and ascitic fluid, agar.*

Character of the colonies: The colonies appear as discrete, thin and translucent, moist, round discs. They can be easily removed when touched with a platinum loop and are also easily emulsified.

NOTE:—Dorset's egg medium is an excellent one for keeping up stock cultures for a long period.

Some general remarks about the cultivation of N. meningitidis

(i) It may be mentioned that the culture media should always be prepared as fresh as possible. Sera and hydrocele and ascitic fluids which have been kept for a long time, do not appear to favour good growth.

(ii) A medium must always contain sufficient water of condensation, as a dry surface fails to produce any growth.

(iii) Blood agar, blood-smeared agar, hydrocele or ascitic fluid agar are better media than serum ones.

(iv) All culture media should remain warm at the time of the inoculation. Cold inhibits the growth.

(v) For the detection of the carrier condition, plate culture is the best.

(vi) Although N. meningitidis grows in a medium containing fresh albuminous fluid, a growth can be obtained, sometimes, in an ordinary agar. For this purpose, a large volume, say 3 to 4 c.c., of freshly drawn cerebrospinal fluid from a meningitis case is put into an agar slant and incubated. A thin film of growth takes place in the agar just above the fluid level. Shaking disturbs this growth.

(vii) Small quantities of nasal mucus are said to enhance the growth of N. meningitidis, while saliva inhibits its growth, due to the presence of salivary bacteria, specially the Streptococci.

(viii) Sodium chloride acts as a poison to N. meningitidis, whereas calcium and potassium chlorides favour its growth.

BIOCHEMICAL REACTIONS:—It ferments glucose and maltose, with the production of acid but no gas. It does not ferment saccharose. Fermentation tests can be carried out either in Hiss's serum water medium (p. 72) or in a solid medium, as used for N. gonorrhœæ (p. 265).

VIABILITY:—The organism is very delicate, like N. gonorrhœæ, and is extremely sensitive to changes of temperature and to the action of antiseptics, sunlight and drying. Organisms, both in culture and nature, die and disintegrate readily; hence frequent subcultures are necessary to keep them alive. It has been found that even with repeated subcultures in suitable media, the organisms may die suddenly.

Longevity in cultures—In most culture media, it is not advisable to keep a well-established strain for longer than 10 days, except in Dorset's egg medium where, with waxed tubes, it can live for more than six months.

Longevity in inflammatory exudates—The organism is very intolerant to cooling and drying. Hence, to ensure a successful culture from the spinal fluid of cases of meningitis and from the nasopharyngeal secretion of "cases" and "carriers," certain precautions are necessary. Either the material is inoculated immediately it is collected, or if the patient is at a distance from the laboratory, the plate or the slopes may be taken in a warm jacket and inoculated on the spot. Delay in making cultures often results in failure. Netter and Debré (1911) found that fluids rich in organisms gave only sparse colonies after standing 6 to 10 hours at room temperature and were invariably sterile after 18 hours.

When cultivating directly from spinal fluids, it is advisable to inoculate the plate heavily (1 to 2 c.c.), since many of the cocci in the exudate will fail to develop colonies, possibly because of their prolonged exposure either to body fluids or to their own products in a closed space. A convenient and useful method of obtaining successful cultures is to inoculate a portion of the plate heavily with the material and incubate it at 37°C. for a few hours and then to spread the material over the entire surface of the medium. The cerebrospinal fluid may similarly be incubated for some time before inoculation into culture medium. This process always increases the percentage of positive cultures.

VIRULENCE:—In artificial media, the degree of virulence of N. meningitidis varies very much. It depends on the time elapsed in making primary cultures from the cerebrospinal fluid, as a few hours' delay will result in the production of colonies which are less virulent than those obtained immediately. Then again, the medium in which a culture is maintained is also important. The experimental exaltation of virulence of the organism, though not always constant, is best carried out by the intra-peritoneal inoculation of mice. *Murray and Ayrton* made an interesting observation that the virulence of the organism can be raised *in vitro* by the addition of an extract of polymorphonuclear leucocytes.

TOXIN PRODUCTION:—No exotoxin has been demonstrated in cultures, but the organism possesses a very powerful endotoxin which is liberated when the organism undergoes disintegration.

SEROLOGICAL REACTIONS:—When Neisseria meningitidis is injected into animals for immunising purposes, the serum of such animals develops the property of agglutinating the homologous cocci and finally causing their dissolution. It is in this way that several varieties of strains have been isolated. For preparing the immune sera (agglutinating sera), young rabbits may be inoculated intravenously with the organism; usually 2 to 3 injections of 500 to 1000 millions of killed bacteria in one day at interval of one hour, followed by 3000 millions of the cocci 6 days later, will suffice to produce a *titre* of above 1 : 1,000 in about 9 days.

The following antibodies appear in the blood of a patient suffering from the disease, when life is prolonged for a sufficient length of time:—(i) *agglutinins*, (ii) *specific opsonins* and (iii) *complement-fixing bodies*. Thus, an agglutination reaction which is obtained with the patient's serum may be used in the diagnosis of the infection.

VARIETIES:—*Gordon*, by applying agglutination and agglutinin absorption reactions, has been able to differentiate several varieties of N. meningitidis. Altogether four have been recognised and are called Types I, II, III and IV. Antigenic structure of the individual strain is complex, as is shown in Fig. 156. It will be seen that Types I and III appear to be closely related, as also Types II and IV. Some minor degrees of relationship, however, exist between the various types, except between the Types I and IV which are absolutely unrelated.

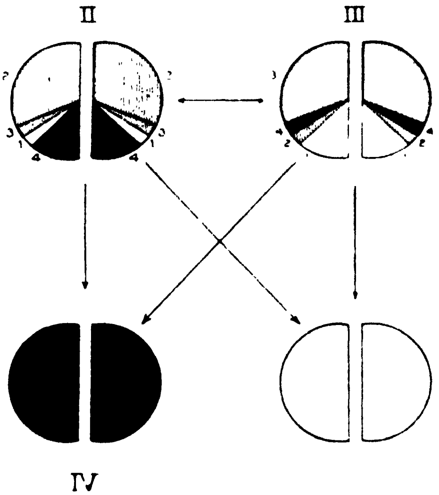


Fig. 156.

Diagrammatic representation of the antigenic structure of various types of *Neisseria meningitidis*. The comparative relationship between the different types, is shown by arrows. (Modified from Fildes and Baker's illustration).

ANIMAL INOCULATION :—When inoculated intraperitoneally in fairly large doses, it is pathogenic to mice, rats and guinea-pigs; rabbits are extremely resistant. There is no true infection, but the animals die from toxaemia in 1 to 4 days. The organisms do not multiply inside the body of the animal. Thus, it will be seen that animals are not very susceptible to infection with N. meningitidis and the characteristic disease, as seen in man, is not produced in animals at all. Recently, *Flexner* has, however, been able to produce meningitis in monkeys by intraspinal and intracerebral injections of pure cultures of the organism.

A comparison between N. meningitidis and N. gonorrhœæ

Neisseria meningitidis resembles *Neisseria gonorrhœæ* in many respects, particularly in its appearance, staining reactions, cultural characters and viability. The differences between the two organisms, however, are shown in the following table.

	N. gonorrhœæ.	**N. meningitidis.**
Morphology	Adjacent surfaces concave.	Adjacent surfaces flattened.
Cultural characters	Dysgonic (difficult to grow). Growth is slow. Colonies are viscid and difficult to emulsify.	Eugonic (more easily grown). Growth is rapid. Colonies are easily emulsifiable.
Biochemical properties	Ferments (acid without gas formation) glucose only.	Ferments (acid without gas formation) glucose and maltose.
Toxicity ...	Slightly toxic to animals.	Highly toxic to animals.

PATHOGENICITY

N. meningitidis does not produce any pathogenic lesions *in animals*.

In *HUMAN BEINGS*, it produces two most important conditions, viz., (i) Nasopharyngeal catarrh (rhino-pharyngitis) and (ii) Cerebrospinal fever with meningitis.

Mode of infection:—Most of the cases (95 per cent.) acquire their infection by contact with the so-called " healthy " or " primary " carriers who are potentially infective. The two most important factors which help in the spread of the disease are (i) over-crowding and (ii) increased capacity of spraying the germs during a catarrhal condition of the nasopharynx. It should be noted in this connection that transmission of germs, from one man to another sleeping in the next bed, occurs up to a distance of about 3 feet and hence, by spacing out the sleeping beds to a distance of 3 feet, the spread of infection can, to a certain extent, be minimised.

Carriers:—These are persons with infected nasopharynx. They may be primary and secondary.

(a) *PRIMARY CARRIERS* are those who have not developed meningitis. These fall under two groups—

(i) *Contact Carriers*, when they have been associated with a case of meningitis. Their rate is higher than that of non-contacts.

(ii) *Non-contact Carriers*, when they have not been in contact with a case of meningitis. These are the persons in whom N. miningitidis produces a primary nasopharyngeal catarrh (rhino-pharyngitis), without the development of meningitis and other secondary lesions.

(b) *SECONDARY CARRIERS* are those in whose nasopharynx the organisms persist after their recovery from the more serious condition, viz., meningitis.

In most cases, the condition of primary carrier does not persist for more than six weeks and only in 10 per cent. of the cases it may persist longer, when they are regarded as chronic carriers. In about 2 per cent. of the cases, it persists for six to twelve months or even more.

Portal of entry:—Cerebrospinal fever is an air-borne disease and is obtained, by " droplet method," directly from an individual who happens to harbour the organisms in his throat. The latter gain entrance into the nasopharynx where they first localise, causing a catarrhal inflammation of the part.

Infection with Neisseria meningitidis: This can be described under three stages as follows:—

First stage—Infection of the nasopharynx (Primary nasopharyngeal catarrh).

Second stage—Generalisation of the infection (Septicæmia).

Third stage—Localisation with the formation of secondary or metastatic lesions.

Stage of infection of the nasopharynx (Primary nasopharyngeal catarrh)

Having gained entrance into the nasopharynx, the organisms produce hyperæmia and a catarrhal condition of the mucous membrane. In the majority of cases, the lesions do not progress and the cocci tend to die out in about three weeks' time. Persons so infected are often regarded as " primary carriers (non-contact)." Elimination of the germs from the throat is effected by the primary defences of the body, such as phagocytosis, lysis by leukins and bacterial antagonism. Apart from generalisation, extension of the infection into the accessory air sinuses of the nose, producing suppurative sinusitis, has been described.

Stage of generalisation of the infection (Septicæmia)

Owing to an unusually increased virulence of the organisms and also due to diminished resistance and high degree of susceptibility, the cocci may get into the general circulation through the inflamed and vascular submucosa and are then disseminated throughout the body. At this stage, there may be a fulminant septicæmia without meningitis. In most cases, the organisms show a tendency to localise in various tissues, organs and fluids of the body, particularly the subarachnoid spaces. Skin rashes (petechiæ) which are found in this disease are due to an embolism of the smaller arterioles by the circulating organisms.

Stage of localisation with the formation of secondary or metastatic lesions

These are *meningitis*, *endocarditis*, *arthritis* and *localised abscesses*.

Meningitis—The organisms show a special affinity for the subarachnoid space, where they excite an inflammatory reaction, causing what is called an **acute cerebrospinal lepto-meningitis**. The following factors render the subarachnoid space a suitable habitat for the Neisseria meningitidis:—

(i) Absence of opsonins and complements in the cerebrospinal fluid.

(ii) Impenetrability of this space to circulating antibodies in the blood.

(iii) Interference, during infection, with its normal drainage by perivascular cellular infiltration.

There appears to be a good deal of controversy as to how the pia-arachnoid membranes are involved. The French observers, chief of whom are Netter and Debré, believe that the germs reach the meninges, either through the cribriform plate of the ethmoid bone along the branches of the olfactory nerves, or through the lymphatics in cases of suppurative inflammation of the accessory sinuses of the nose, specially the sphenoidal sinuses. On the other hand, many authorities in Germany, America and Great Britain, consider that the involvement of the meninges is due to a primary septicæmia with secondary localisation in this area. It may be pointed out in this connection that in cases of catarrhal inflammation of the naso-pharyngeal mucous membrane, the barrier of natural resistance is very much weakened, as a result of which, pathogenic micro-organisms, such as Diplococcus pneumoniæ, Streptococci, Hæmophilus influenzæ and also the Neisseria meningitidis, get a chance to invade the blood stream through the dilated blood and lymphatic vessels. This may explain the most fulminant cases of cerebrospinal fever in which patients die of a virulent septicæmic condition, but where the characteristic lesions are hardly noticeable in the brain and meninges. From a very large amount of clinical and bacteriological experience, we believe that the N. meningitidis gain access to the subarachnoid spaces of the brain and spinal cord via the blood stream.

When the pia-arachnoid membranes are once reached, the organisms produce a very intense and acute inflammatory process in the meninges, as well as in the brain. The lesion may [illegible] be called a true *meningo-encephalitis*. Such a lesion is associated [illegible] an inflammatory exudate, consisting of a copious sero-fibrinous mat[illegible]tangling in it innumerable polymorphonuclear leucocytes (Fig. 157). This at first appears at the base of the brain but subsequently it spreads to the lateral surfaces, as well as to the vertex of the cerebral hemispheres where the exudate is often very thick. The spread of the exudate from the base towards the vertex and its concentration at the latter situation may be explained by the fact that normally the current of the cerebrospinal fluid passes from the base upwards to be absorbed by the arachnoideal granulations (Pacchionian bodies). In a typical case, the entire surface of the brain and spinal cord become with a thick sheet of fibrino-purulent material, with the intensely engorged and dark pial vessels standing out in marked contrast and all the sulci entirely obliterated (Fig. 158).

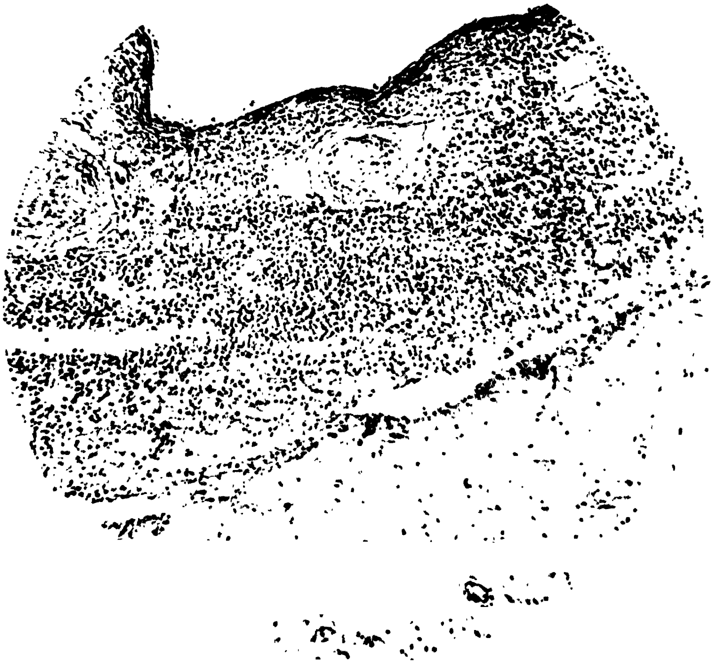


Fig. 157.

Photomicrograph of Fig 158, showing exudate in the

As there is a serious interference with the normal drainage of the cerebrospinal fluid by the inflammatory exudate, there is always an increase in its volume and tension. This produces flattening of the convolutions and the ventricles tend to be over-distended with the turbid fluid. The substance of the brain is soft and diffluent and when cut, appears to be very much congested.

In some cases, the exudation is almost entirely confined to the base of the brain, and specially to the under surface of the cerebellum and pons, extending forwards as far as the optic chiasma and often affecting the tips of the temporo-sphenoidal lobes. It is for this latter distribution that the disease was named "**posterior basic**" **meningitis.** This condition, which was first described by *Gee* and *Barlow* in 1878, occurs principally in infants between the ages of 6 months and 2½ years, and is characterised by a very marked retraction of the head and the early development of hydrocephalus. The character of the cerebrospinal fluid, the nature of the micro-organisms and the morbid anatomy of the disease do not, however, differ materially from the acute meningococcal meningitis of the adult.

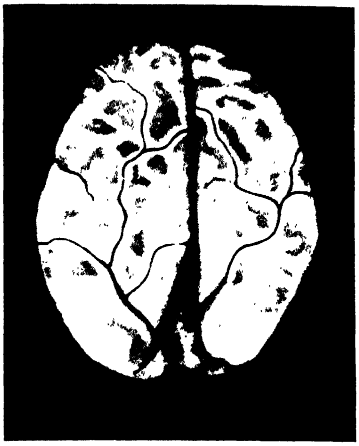


Fig. 158.

Gross appearance of a brain from a case of cerebro-spinal fever. The whole surface of the brain is covered with a thick sheet of fibrino-purulent exudate, obscuring the sulci and gyri The dark lines represent the meningeal vessels.

Endocarditis—This is due to a localisation of the organism in the cardiac valves. The left side is affected most and a vegetation, similar to that of Neisseria gonorrhoeæ or Diplococcus pneumoniæ, is formed on the affected valves. Endocarditis usually occurs some time after the acute stage is over and in a patient who survives for a long time after meningitis. It is, after all, a very rare occurrence.

Arthritis—Sometimes, the synovial membranes and the periarticular tissues are invaded by the organisms, producing an acute arthritis, with or without suppuration. It does not differ from the other forms of suppurative arthropathies caused by pyogenic cocci. The organisms may be recovered from the joint fluid and pus.

Localised abscesses—Occasionally, the N. meningitidis, like other pyogenic cocci, may produce miliary abscesses in internal organs, as well as under the skin. The incidence of such a condition, however, is very rare.

Characters of the cerebrospinal fluid

1. **Physical**: Both the pressure and the amount of the fluid are considerably increased. In some cases, it may come out through the puncture needle in a jet, the quantity exceeding 100 c.c., before the normal pressure and flow are restored. On an average, this quantity is about 30 to 40 c.c.

2. **Gross appearance of the fluid**:—This varies from a slight opalescence to a marked turbidity. It is commonly somewhat clear at an early stage of the infection, but becomes increasingly purulent as the disease progresses. It tends to become clearer again, if the patient survives longer and the disease becomes chronic. A slight green colour may be found in the purulent fluid, due to the presence of altered hæmoglobin and when allowed to stand, the fluid may clot.

3. **Chemical**: There is a marked increase in the amount of albumen which may rise from a trace in the normal condition to as much as 0·2 to 0·3 per cent. The globulin content also becomes high and Noguchi's butyric acid test is positive. The disappearance of the Fehling-reducing substance is also an important feature.

Noguchi's butyric acid test—To one part (0·2 c.c.) of the fluid are added five parts (1 c.c.) of a 10 per cent. solution of butyric acid in normal saline and heated to boiling. One part (0·2 c.c.) of normal caustic soda is then added and the mixture is allowed to stand for 5 to 10 minutes. A positive reaction is indicated by the formation of a heavy white coagulum. A faint but definite opalescence is, however, normal.

4. **Cytological**:—Polymorphonuclear leucocytes form the main bulk of the cellular elements, reaching as high as 99 per cent. If the disease is chronic, there may be an increase in the number of lymphocytes and other mononuclear cells. The presence of intact red cells always indicates an injury to vessels during lumbar puncture.

5. **Bacteriological**:—Usually, when a film is prepared from the turbid fluid or from the centrifugalised deposit, and stained with the usual stains, the typical N. meningitidis are seen. They may be intra or extra, cellular in position, depending on the stage and intensity of the infection, as well as on the virulence of the organisms. The cocci may not be found (1) if the disease becomes chronic, (2) if the fluid is allowed to stand outside for a long period or (3) if specific serum therapy has been carried out. After smear examination, a culture of the fluid in the usual way will reveal the characteristic growth of the organisms.

THERAPEUTIC CONSIDERATIONS

Vaccines:—The use of vaccines in cases of epidemic cerebrospinal fever has not been attended with much success. This is obvious from the fact that the disease process is very acute and the infecting germs are intensely toxic. The use of vaccine-therapy in the treatment of carriers has been equally unsuccessful. Prophylactic immunisation with vaccines of N. meningitidis has been tried but without much success. The antibodies which appear in the blood do not reach the cerebrospinal fluid.

Antiserum:—Injections of antiserum in a case of meningococcal infection of the blood and meninges have been attended with very marked benefit. Since its introduction in therapeutics by Flexner, the case mortality has fallen from *70 to 80 per cent. to about 30 to 40 per cent.* Besides *lowering* the incidence, the administration of antiserum has definitely reduced the incidence of relapse and chronicity of the disease. Another well marked advantage which is found, after treating a case with antiserum, is the marked diminution in the rate of development of complications. The action of the serum is probably due to the presence in it of opsonins, agglutinins and complement-fixing bodies, and not due to any direct antitoxic or bactericidal effect. Its injection is followed by an increased phagocytosis and diminution and disappearance in the number of cocci in the cerebrospinal fluid.

Mode of administration and the quantity are the important factors to determine the efficacy of the antiserum. It is also essential that a polyvalent serum be used and administered as early as possible. To obtain the maximum effect, both intrathecal and intravenous injections should be made.

To start with, a lumbar puncture should be done and the excess of cerebrospinal fluid drained off, until the rate of flow is about 60 drops per minute. Twenty to thirty c.c. of anti-meningococcal serum at body-heat are very slowly injected through the puncture needle or preferably allowed to run in by gravity. About 40 to 60 c.c. of the serum are also injected intravenously. The process should be repeated at intervals of 8 to 12 hours, according to the gravity of the case, for at least 4 to 5 days successively.

PROPHYLAXIS:—As already mentioned, the disease spreads from carriers to normal persons, specially under conditions which lead to overcrowding and lack of ventilation. Hence, the essential step in limiting the spread will be to prevent overcrowding, particularly at night, to ensure free ventilation and to encourage an open air life as far as possible. Prophylactic vaccinations have been tried but without much success.

LABORATORY DIAGNOSIS

A. Of epidemic cerebrospinal meningitis.

Except during an epidemic, it is difficult or impossible to make a diagnosis of this disease without a laboratory examination of the cerebrospinal fluid. Materials usually examined for the purpose are, in the order of their importance, *cerebrospinal fluid*, *blood* and *nasopharyngeal discharge.*

Cerebrospinal fluid:—The material is obtained by means of a lumbar puncture and collected in a sterile test tube. It is then subjected to the following examinations:—

(a) *GENERAL EXAMINATION*—This includes an investigation of the physical, chemical and cytological changes in the fluid, which have already been described.

(b) *BACTERIOLOGICAL EXAMINATION*—

(i) *Microscopical*—A smear from a centrifugalised deposit is stained by Gram's method for the presence of Gram-negative intracellular diplococci which may be considered diagnostic of an infection with N. meningitidis.

As *mentioned before*, the position of the cocci may be both intra and extra, cellular. The extra-cellular position of the cocci, i.e., free in the fluid, is associated with the increased virulence of the strain and this is particularly so in the most severe cases. In such conditions, the spinal fluid may be almost clear, having very few cells but rich in cocci which may be so abundant as to be seen in every field of the microscope.

(ii) *Cultural*—Cultures are made on serum agar or blood agar and suspected colonies are recognised by their specific characters. They are then picked off and their indentification is confirmed by the following:—

1. Biochemical reactions (Fermentation of glucose and maltose).
2. Agglutination reactions with [illegible] meningococcal serum.

For the latter test, a 24 hours' growth is emulsified in sterile saline and standardised to contain 2,000 to 4,000 million cocci per c.c. Macroscopic agglutination is carried out usually with four dilutions, 1 in 50, 1 in 100, 1 in 200 and 1 in 400. The tubes are kept in the water-bath at 55°C. for 24 hours. Preparation of agglutinating sera has already been described.

Serological grouping of any strain may be identified, if the type antisera are available.

Note:—To obtain successful cultures from cerebrospinal fluid, certain precautions are to be taken and these have already been mentioned on pages 276 & 277.

Blood:—

1. *BLOOD COUNT*—An increase of the polymorphonuclear leucocytes is always associated with infection of the meninges by N. meningitidis. Although this may prove of assistance in the diagnosis, it is not specific and such a picture is found equally well in pneumococcal and other pyococcal infections of the meninges.

2. *BLOOD CULTURE*—As the disease is a septicaemia, specially in the early stage, isolation of the organism from the blood may be possible (though very difficult), particularly in fulminant septicaemic cases.

3. *AGGLUTINATION REACTION*—This is useful only at a later stage, if the patient survives. The agglutinins developed in the serum may be demonstrated by an agglutination reaction with an emulsion of the bacterial growth.

Nasopharyngeal discharge—Vide under the diagnosis of carriers.

B. Of carriers:—Diagnosis of carriers involves (i) isolation of the specific organism from the nasopharynx and (ii) demonstration of specific agglutinins in the blood, particularly of those persons who have recovered from the disease.

Nasopharyngeal discharge—

COLLECTION OF MATERIAL—Swabs should be taken from the postnasal space by means of a curved postnasal swab and special care should be taken not to contaminate the material with saliva. For this purpose West's tube is used.

West's post-nasal swab—It consists of a curved glass tube, containing a flexible wire with the terminal ¾ in. carrying the cotton-wool pledget and bent to fit in the tube. (Fig. 159). The apparatus is sterilised as a whole with the wool plug at each end of the tube.

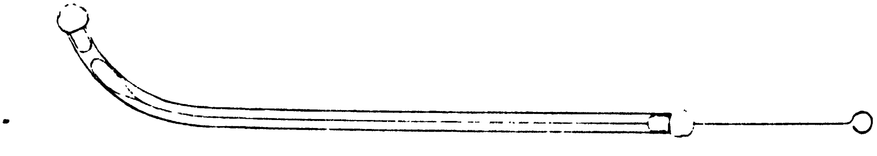


Fig. 159.
West's post-nasal swab.

Method (Fig. 160)—The tube is introduced into the mouth, passed up behind the palate and from this end the swab is pushed on to the roof of the nasopharynx, the tongue being depressed with a spatula. An appreciable pressure is exerted over the pharyngeal tonsil, and any adenoid tissue present is brushed over with the swab, taking away the tenacious mucus and the micro-organisms with it. It is then retracted into the tube and withdrawn from the mouth.

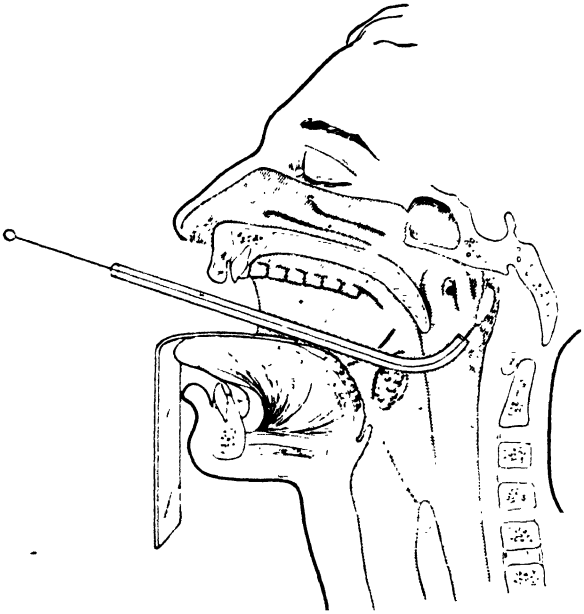


Fig. 160.

Method of taking post-nasal swab.

EXAMINATION OF THE COLLECTED MATERIAL:—This consists of microscopical and cultural examinations which are further confirmed by serological tests. Identification of the organisms in the nasopharynx offers a great difficulty, owing to the presence of a number of other Gram-negative diplococci in that situation. The chief organisms likely to cause confusion are N. catarrhalis, N. flava and N. pharyngis sicca. The following points serve to give a positive diagnosis of Neisseria meningitidis:—

(i) Absence of growth on agar at room temperature.

(ii) Ready emulsification of the colonies in saline.

(iii) Fermentation of glucose and maltose; non-fermentation of saccharose.

(iv) Agglutination by anti-meningococcal serum.

A table has been given on page 280 to show the differences between the various Gram-negative diplococci that are found in the nasopharynx.

OTHER GRAM-NEGATIVE ALLIED DIPLOCOCCI

The group includes the following:—

1. Neisseria catarrhalis (M. catarrhalis).
2. Neisseria flava (M. flavus).
3. Neisseria sicca (M. pharyngis sicca).
4. Diplococcus crassus.
5. Diplococcus mucosus.

Habitat:—They are commonly met with in the nasopharynx.

Morphology:—All the organisms have a close resemblance to N. meningitidis. D. crassus is rather larger than N. meningitidis and, especially in subcultures, may tend to assume Staphylococcal forms. Capsules are present in D. mucosus.

Staining reactions:—All of them are Gram-negative, with the exception of D. crassus which shows a marked variation in its Gram staining character, as some of these cocci may retain the Gram's stain (Gram-positive).

Culture:—They grow well at the room temperature (22°C.), both in the tropics as well as in the temperate climates, and can be cultivated easily in ordinary media, agar and broth.

CHARACTER OF COLONIES:—

N. catarrhalis—Colonies closely resemble those of N. meningitidis, but are larger and more opaque. Colonies are extremely tough and do not emulsify easily. They can, however, be easily picked up from the media. When touched with a platinum loop, the whole colony is removed.

N. sicca—Colonies are very tough and dry and adhere to the medium.

N. flava—It is chromogenic and develops a yellowish colour after 48 hours' incubation. Three varieties of this organism have been described.

D. crassus—Colonies are small and resemble those of streptococcus.

D. mucosus—Colonies are of a slimy consistency.

Biochemical reactions:—See the table on p. 289.

Serological reactions:—None of them are agglutinated by antimeningococcal serum.

Pathogenicity:—Most of them are devoid of any pathogenic property, with the exception of the N. catarrhalis which produces nasopharyngeal catarrh.

TABLE SHOWING THE DIFFERENTIATION OF VARIOUS GRAM-NEGATIVE COCCI.

Organism.	Growth at 20°C.	Growth on ordinary agar.	Glucose.	Maltose.	Saccharose.	Pathogenicity.		Source.
						Animal.	Man.	
N. gonorrhœæ ...	o	o	+	o	o	Low	Gonorrhœa.	Urethral discharge.
N. meningitidis ...	o	o	+	+	o	Low	Cerebrospinal fever.	Nasopharyngeal discharge, cerebrospinal fluid and other meningococcal exudates.
N. catarrhalis ...	+	+	o	o	o	Nil	Nasopharyngeal catarrh.	Nasal and pharyngeal discharge.
N. sicca ...	+	+	+	+	+	Nil	Nil	Nasopharynx.
N. flava ...	+	+	+	+	o	Nil	Nil	Nasopharynx.
D. crassus ...	+	+	+	+	+	Nil	Nil	Nasopharynx.

+ = Positive reaction; o = No reaction.

Diplococcus mucosus is differentiated from all the members of this group by the presence of a capsule and production of mucoid colonies.

CHAPTER XXIII

ACID-FAST ORGANISMS (MYCOBACTERIA)

The acid-fast organisms are so called, because of the property they possess, when stained with basic fuchsin, of retaining the red colour after being treated with dilute mineral acids. This property appears to be due to the existence of a lipoid envelope which surrounds the bacterial body and resists the action of the acid on the stain.

The following are the common acid-fast organisms:—

I. ***Pathogenic to man and animals:***—

1. **Mycobacterium tuberculosis** (B. tuberculosis)—The causative organism of tuberculosis in animals (bovine type) and man (human type).
2. **Mycobacterium lepræ** (B. lepræ)—The causative organism of human leprosy.
3. **Mycobacterium lepræ murium**—The organism of rat leprosy.
4. **Mycobacterium paratuberculosis** (Bacillus of Johne's disease) The causative organism of chronic enteritis of cattle and sheep.

II. ***Non-pathogenic to man*** (having a saprophytic existence):—

5. **Mycobacterium smegmatis** (B. smegma).
6. **Mycobacterium butyricum** (The Petri-Rabinowitsch butter bacillus).
7. **Mycobacterium phlœi** (Mœller's Timothy-grass bacillus).

MYCOBACTERIUM TUBERCULOSIS

(Bacillus tuberculosis)

The causative organism of tuberculosis.

HISTORICAL:—In the study of the subject of tuberculosis three great names are worth mentioning:—

Lænnec (Fig. 161), a French clinician, who himself was a consumptive and succumbed to the disease, gave the first scientific and accurate description of the "tubercle" and the various other lesions of 'consumption" in 1819.

In the year 1865, **Villemin** (Fig. 162), a French Military Surgeon, proved for the first time that 'tubercle' is a specific affection and its cause is an inoculable agent.

Fig. 161.
R. T. H. Laennec (1781-1826)
(From Calmette's tuberculosis; by kind permission of Williams and Wilkins Company).

Fig. 162.
J. A. Villemin (1827-1892).
(From Calmette's tuberculosis; by kind [illegible] *of Williams and Wilkins Company).*

The successful discovery of the real etiological agent, viz., the Mycobacterium tuberculosis, was reserved for a German Physician, **Robert Koch** (Fig. 163), who, in 1882, demonstrated and successfully cultivated the causative organisms from tuberculous material. He was also able to reproduce the disease in animals by inoculation methods. In the same year, **Ehrlich** discovered the peculiar acid-fast character of the organisms.

Fig. 163.
Robert Koch (1843-1910).

HABITAT:—Mycobacterium tuberculosis is strictly a parasitic organism. The *human type* is not encountered as a normal inhabitant in the human body. It is, however, found in persons suffering from tuberculosis. The *bovine type* of infection in human beings is obtained from diseased cattle, usually through infected milk.

VARIETIES:—

[illegible] *Mycobacterium tuberculosis h*[illegible] The causative organism of tuberculosis in man.

2. *Mycobacterium tuberculosis bovis*—The prevalent organism in animal tuberculosis and also responsible for a small percentage of human tuberculosis, specially in children.

3. *Mycobacterium avium.*

4. *Mycobacterium piscium.*

MORPHOLOGY:—It is a rod-shaped organism, measuring about 2 to 4 μ in length and 0·2 to 0·5 μ in breadth. In stained preparation, the organisms often show *a beaded or granular appearance*. The former is due to the presence of one or more unstained or slightly stained intervals and is considered to be due to the segmentation of the chromophil substance; the latter is due to the presence of deeply staining granules (chromatin granules).

It is a *non-flagellated, non-motile* and *non-sporing organism*. It does not possess a capsule but the bacterial body is provided with a *lipoidal envelope*.

STAINING REACTIONS:—Ordinary aniline dyes fail to stain the bacilli satisfactorily, the lipoid envelope resisting the penetration of the dyes. Differential stain (p. 14), such as Ziehl-Neelsen's technique, is usually employed. This is the classical stain and by this method (p. 22), the bacilli are stained well and when once stained, they resist decolorisation by dilute mineral acids (acid-fast) or alcohol (alcohol-fast). This staining peculiarity (Fig. 164) renders it possible to make a positive bacteriological diagnosis

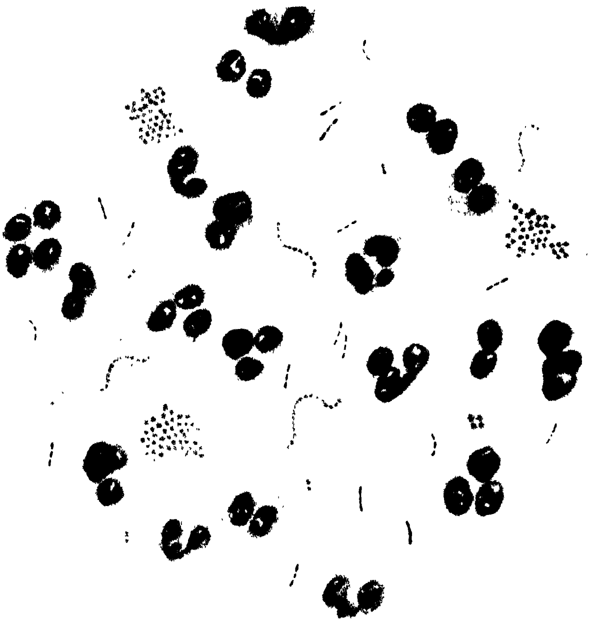


Fig. 164.

Sputum stained by Ziehl-Neelsen's method, showing the acid-fast character (red colour) of *Mycobacterium tuberculosis*. Malachite green has been used as the counter-stain.

in the majority of cases by the microscopic examination of a stained smear only. When stained by Gram's method, it is Gram-positive.

CULTURAL CHARACTERS:—The following points should be remembered in connection with the cultivation of the organism:—

1. Cultivation of Mycobacterium tuberculosis in ordinary media is absolutely useless, special media being therefore necessary.

2. Growth of the organism is very slow. It takes about 2 to 3 weeks before any growth takes place. For this reason, the inoculated tubes are sealed with paraffin to prevent evaporation.

3. Plating method for isolation cannot be used, because by the time the M. tuberculosis will develop, the plate is overgrown by other contaminating organisms.

4. Glycerin (5 to 8 per cent.) and albuminous materials promote greatly the growth of the bacilli, particularly of the human type.

5. It grows best aerobically at a temperature of 37°C. To allow a free access to the oxygen of the atmosphere, the sealed tubes should be opened from time to time.

6. It resists the action of antiformin (10 to 30 per cent.) or caustic soda (3 per cent.).

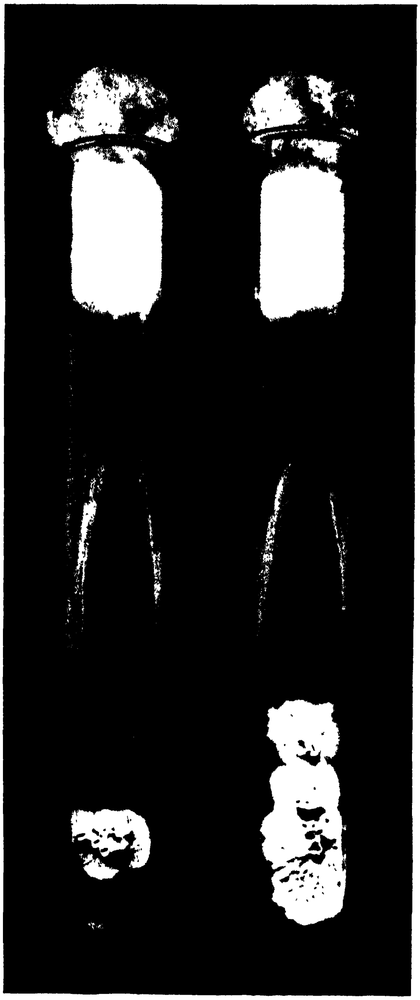
Media for the growth of M. tuberculosis:—

A. **For primary culture**:—Culture of M. tuberculosis may be obtained direct from a tuberculous material with certainty on solid media containing glycerin or albuminous material. Before inoculation, however, the material, if contaminated with secondary micro-organisms, must be treated with antiformin (*vide* p. 317). For primary culture, the following media are very useful: *Dorset's egg medium, inspissated blood serum medium and Petroff's medium* (p. 65).

Character of the growth:—The human type grows luxuriantly (eugonic) in all media and may form a wrinkled film (Fig. 165) or a verrucose (mamillated) growth (Fig. 166) which is irregular, tough and looks dry. The growth is not readily emulsified. It is at first white but later becomes buff-coloured. The bovine type, however, grows scantily (dysgonic) and primary culture on glycerinated media often fails to show any growth.

B. **For subculture,** the following media are very useful—*coagulated serum medium* and *Dorset's egg medium.*

C. **For mass cultures**—The medium commonly used is *glycerin broth.* This is usual nutrient broth into which 5 to 6 per cent. of glycerin has been added. The bacilli form a white wrinkled pellicle, which spreads on the surface of the medium (Fig. 167). This mode of growth is suitable for the production of tuberculin. *Glycerin potato medium* (p. 65) may also be used for this purpose.



Figs. 165 and 166.

Growth of *Mycobacterium tuberculosis* on solid media.
A—Bovine type, B—Human type

VIABILITY:—Acid-fast bacilli, while very susceptible to heat, are very resistant to chemical disinfectants. For instance, the bacilli are killed when exposed to moist heat at 60°C. in 15 to 30 minutes. But they may survive the action of 5 per cent. carbolic acid for several hours. Thus, in their susceptibility to heat, they resemble other non-sporing bacteria, while in their resistance to chemical disinfectants, they resemble the spore-bearing organisms. When exposed to a temperature of 95°C. in milk, they are killed in 1 minute. This is very important from the public health point, as by this means, the infective power of a tuberculous milk is destroyed. The bacilli can withstand the action of gastric juice and hence, when ingested, can pass on to the intestinal canal where they produce their characteristic lesions.

Another point to be remembered is that the organism can survive putrefaction and its staining character (acid-fast property) is not altered in any way. Microscopic examination of a stained smear of such decomposed materials will enable one to give the diagnosis of M. tuberculosis.

Sunlight and desiccation have a very important bearing on the vitality of the M. tuberculosis. Direct sunlight is very injurious to the bacilli. In dried sputum, the bacilli may retain their viability for weeks and months, specially when protected from strong light. This point should be borne in mind, as such a dried infected sputum is the commonest source of tuberculous infection. These conditions are more favourable in dwelling houses than in open places and it is for this reason that the open-air treatment and an outdoor life are specially advised for a tuberculous patient, as by this means, not only the general resistance is increased but the infectivity of a case is also minimised.

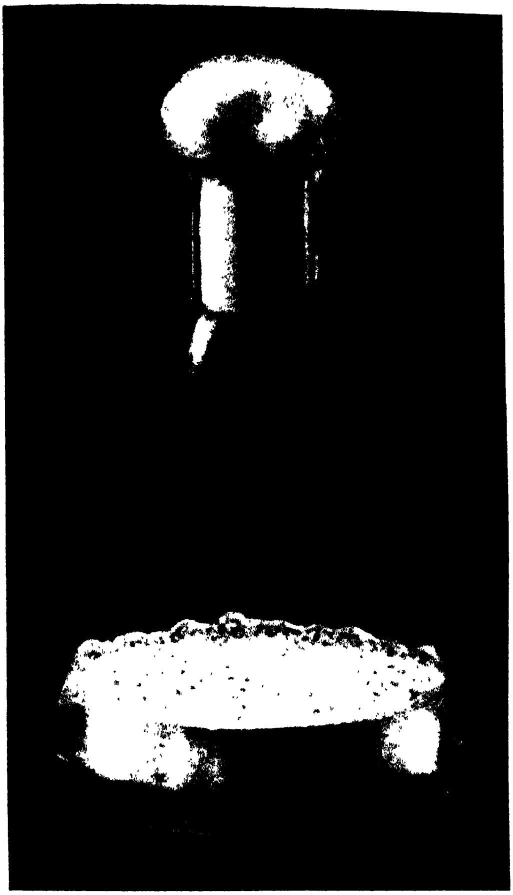


Fig. 167.

Growth [illegible] M *tuberculosis hominis* in glycerin broth [illegible] wrinkled film of surface growth which has spread up th[illegible] of the flask

It has already been mentioned that M. tuberculosis resists the action of certain chemical agents, e.g., (i) antiformin, 10 to 30 per cent., (ii) caustic soda, 3 to 5 per cent. and (iii) gentian violet, 1 in 10,000 dilution. This knowledge is utilised in isolating the organism from materials contaminated with secondary organisms (*vide* antiformin and Petroff's method) and also in the preparation of culture media (Petroff's media).

TOXIN PRODUCTION: The bacilli do not produce any exotoxin. The toxic substances are usually derived from the disintegration of the bacilli themselves and thus only *endotoxins* are available. Preparations consisting of suspensions or extracts of such disintegrated products of Mycobacterium tuberculosis are called *tuberculins* which are exactly the same, whether prepared from a human or a bovine strain. That obtained from the human strain is designated as T and the one from the bovine strain as PT.

Varieties of tuberculin: —There are various methods of preparing tuberculin, but the two most commonly employed for diagnosis and treatment are the following: —

1. **Koch's old tuberculin** A good strain, either a bovine or a human one, is chosen and is allowed to grow as a surface film in flat-bottomed flasks,

containing 5 per cent. glycerin broth. After 6 to 8 weeks' cultivation at 37°C., the bacilli are killed by heat at 115°C. in the autoclave for half an hour and the culture is filtered through a porcelain filter. The filtrate which is then evaporated over a water bath at 80°C. to one-tenth of its original volume, constitutes the old tuberculin and contains disintegrated bacillary bodies, soluble products of bacteria not destroyed by heat and substances derived from the medium. The preparation is then standardised and its potency is compared with that of a standard product, on tuberculous guinea-pigs (infected 3 or 4 weeks previously).

2. **Koch's new tuberculin (Bazillen emulsion or B.E.)**—Masses of living bacilli, obtained from cultures on solid medium or a film from fluid medium, are dried *in vacuo* and then ground up in a mechanical mill, until no intact acid-fast bacilli can be found on microscopic examination. This is then emulsified in 50 per cent. aqueous glycerin, so that 1 c.c. of the fluid contains 5 mgm. of the bacillary substance. This preparation corresponds to a bacterial vaccine.

Chemical nature of tuberculin:—So far it has been analysed into two portions—(i) a carbohydrate moiety which reacts with the antibodies *in vitro* and (ii) a protein residue which is responsible for the tuberculin effects.

Uses of tuberculin:—This may be used for two purposes, (i) as an aid to diagnosis (*vide* p. 320) and (ii) as a therapeutic agent (*vide* p. 314).

Tuberculin does not produce any ill-effects in non-tuberculous body and it is only in the tuberculous animal that it appears to be toxic. It will be mentioned later that tuberculous animals develop a supersensitiveness to tuberculin. Hence, when toxins of the M. tuberculosis are injected into susceptible animals, they manifest this hypersensitiveness in the following ways:—

1. *A local reaction*—This consists of an inflammatory swelling produced at the site of the inoculation.

2. *A general reaction*, indicated by malaise and pyrexia of varying degrees.

3. *A focal reaction*—This is an exacerbation of the inflammatory reaction produced around the tuberculous lesions, and is manifested by an increase in the local signs and symptoms of the disease. This focal reaction, by producing increased vascularity and cellular response, might lead to the encapsulation of the tuberculous lesion, producing a cure; or if the lesion is superficial and visible, e.g., in lupus, the latter may be ulcerated and cast off.

These reactions, accordingly, supply some valuable means by which the presence of tuberculous foci in the body can be recognised. From the results of the focal reaction, it will be observed further, that tuberculin can be applied both for therapeutic as well as for diagnostic purposes. The employment of tuberculin may thus act as a stimulant to the tissues in the neighbourhood of the tuberculous lesions and thus be beneficial; it may also aid by leading to an increased production of specific antibodies.

SEROLOGICAL REACTION:—The following antibodies have been demonstrated in the serum of a patient suffering from tuberculosis, such as opsonins, agglutinins, complement fixing bodies. The development of such immune bodies no doubt raises the general immunity to a certain extent, but they do not conduce to a rapid healing. This is because the nature of the histological lesion is such that some of the bacteria are beyond the reach of these protective substances. Hence, attempts to treat tuberculous patients with artificially prepared antisera were unsatisfactory. Some of the serological tests (*vide infra*) are employed in the diagnosis of tuberculous infection but they cannot be relied on too much.

ANIMAL INOCULATION:—

Choice of experimental animals:—Of all the animals ordinarily used in the laboratories, the *guinea-pigs* and *monkeys* are the most susceptible to tuberculous infection, particularly when one is using the bacilli of the human type. The *rabbit* is somewhat resistant but succumbs very quickly to inoculation with the bovine bacilli. One-tenth of a milligramme of a culture of bovine bacilli usually suffices to kill a rabbit in less than two months, while, with the human bacilli, it is, as a rule, refractory to infection by the same path with even 10 mgms.

Methods:—The lesions can be produced experimentally in animals by the following methods—*by injections (intravenous, intraperitoneal and subcutaneous), by inhalation, by ocular instillation and by ingestion.*

1. **By injections**:—The acuteness of the lesions will depend on the dose of the bacilli and the route of inoculation. With intravenous inoculation, acute general miliary tuberculosis is induced in susceptible animals. The typical lesions are found in the spleen, liver, lungs and lymph nodes. Miliary as well as nodular lesions are produced. With intraperitoneal inoculation, the same forms of general tuberculosis are produced as in the case of the intravenous injection, but they are less acute in nature. Local lesions in the peritoneum are also seen. With subcutaneous inoculation of a small dose of bacilli, there is a tendency to a primary localisation at the seat of the injection, with involvement of the regional lymph nodes. Later on, there may be general dissemination by the blood, depending on the number and relative virulence of the infecting organisms, as well as on the species of animal used. The usual site of inoculation is either the lower part of the abdominal wall or the upper and medial aspect of the thighs. The first sign of the disease is manifested about 8 to 10 days later, by the swelling of the nearest lymph nodes.

The usual mode of inoculation, however, is the subcutaneous one, but intraperitoneal methods may also be employed. Intravenous route is not used under ordinary circumstances.

2. **By inhalation**:—Pulmonary lesions are easily induced by this method, and it has been observed that one virulent bacillus is sufficient to cause infection in a guinea-pig. The lesion is visible to the naked eye on about the ninth day. With massive doses, however, the infection becomes generalised.

3. **By ocular instillation**:—This is carried out by the simple instillation of a drop of sputum containing the bacilli or of a culture upon the surface of the conjunctiva. The local eye changes will vary according to the dose of the bacilli instilled. The first apparent manifestation of the infection is the enlargement of the glands in the submaxillary region. The enlargement of the cervical and retropharyngeal glands quickly follows and the latter form a mass in the neck. In guinea-pigs, pulmonary lesions with involvement of the tracheo-bronchial lymph glands and intestinal lesions with involvement of the mesenteric glands may be observed later.

4. **By ingestion**:—This method consists in painting the inside of the cheeks with the infective material, or mixing the bacilli or the bacillus-containing material with food which the animal may be made to feed upon or which may be introduced directly into the stomach by means of a syringe and œsophageal tube. The development of tuberculosis does not always follow this method. When successfully infected, the mesenteric lymph nodes become enlarged and caseous and later on, there may be involvement of the lungs.

The value of experimental tuberculosis—The facts which have emerged from these experimental works on tuberculosis are that, whatever may be the avenue of infection (except intravascular), there is a definite and distinct primary localisation, either at the point of inoculation or in the adjacent lymph nodes, whether the dose is large or small. In conjunctival instillation and feeding experiments, the bacilli may pass through the mucous membrane, without producing any local lesions or leaving any trace of their passage, but the adjoining lymph glands which form the second line of defence are always involved. If the dose of the bacilli is large and the animal is highly susceptible, some of the organisms may readily pass into the general circulation, causing a generalised infection.

PATHOGENICITY OF MYCOBACTERIUM TUBERCULOSIS

Animals:—*Bovine animals* are mostly affected and are responsible for the greater part of the tuberculosis in mammals. Stalled animals are more affected than those which enjoy more freedom. In our country, however, bovine tuberculosis is not so common, but in the British Isles, it may be as much as 25 to 30 per cent. and it is much more so in Scotland.

Apart from all the varieties of lesions occurring in every part of the body, the one which concerns us most is the disease of the mamma (Figs. 168 & 169). The bacilli may be excreted with the milk, not only in a diseased condition of the udder itself, but even without any lesion in it, the disease being situated in other parts of the body. The type of the organism concerned is *Mycobacterium tuberculosis bovis.*

Swine:—These animals are always infected by ingestion through the alimentary canal. The lesions may be caused by all the three types of bacilli but chiefly by the bovine strain.

Other domestic animals, such as the horse, ass, goat, sheep, cat and rat, hardly ever suffer from tuberculosis, except when they live as cohabitants for long periods with tuberculous cattle or man. Rabbits and guinea-pigs, although very susceptible to this infection, do not suffer from spontaneous tuberculosis.

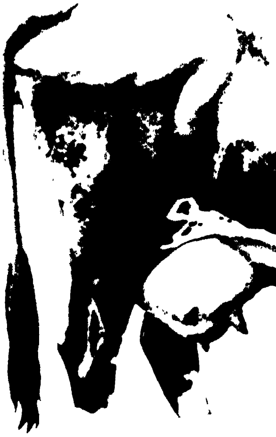


Fig. 168.

Tuberculosis of the mammary gland of a cow.

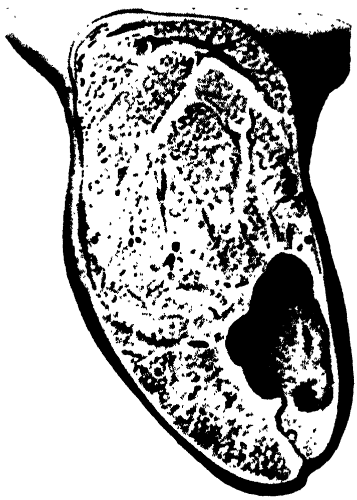


Fig. 169.

Longitudinal section of an udder, containing a tuberculous cavity.

(*From Calmette's tuberculosis; by kind permission of Williams and Wilkins Company*).

Wild animals are never affected with tuberculosis spontaneously, but in captivity, they may be rendered susceptible to it.

Note: The Mycobacterium tuberculosis, obtained from tuberculous lesions in animals, is mostly of the bovine type.

TUBERCULOSIS IN HUMAN BEINGS

Modes of infection and pathogenic lesions:—

A. Pre-natal—Hereditary or congenital transmission of tuberculosis may be considered under the following heads, conceptional (germinative) and transplacental.

1. *Conceptional or germinative* tuberculosis is impossible, because the growth of the Mycobacterium tuberculosis will certainly destroy the infected ovum.

2. *Transplacental route*, though a possibility, is very remote. The bacilli cannot get into the fœtal circulation, unless there is some placental tear.

Rarity of hereditary transmission of tuberculosis is proved by the fact that offsprings of tuberculous parents, if separated immediately after birth from the latter's contact and guarded subsequently against infection, may remain perfectly free from the disease. The part played by heredity in this connection is the production of a ' *diathesis* ' which makes the offsprings less resistant and hence more prone, to the in[illegible]

B. Post-natal:—This is always the case. The M. tuberculosis may enter the system through all the surfaces of the body which are open to invasion from the exterior. They are the following:—*skin, conjunctiva, genito-urinary tracts, alimentary tracts and the respiratory tracts.* Of these, the last two are by far the most important routes for post-natal infection.

1. *SKIN*—Transmission through the skin, though relatively infrequent, sometimes does occur, producing the condition known as "*Lupus vulgaris.*" The skin of the face, nose and the neighbourhood of the anus is particularly liable to be affected. As this condition is most commonly found to be associated with the bovine type of bacilli, the probability for a direct infection is very great. Skin infection may also take place in the post mortem and dissecting rooms, such a condition being used to be called "*Verruca necrogenica of Wilks*" (post mortem warts).

2. *CONJUNCTIVA*—This may be infected by wiping the eyes with a handkerchief soiled with tuberculous sputum (by a nurse), but it is not very common.

3. *GENITO-URINARY*—This method of infection may occur, although it is somewhat uncommon. *Tuberculosis of the cervix uteri* is an important pathological lesion and it is often mistaken for an erosion or a carcinoma.

4. *ALIMENTARY TRACTS*—The common *vehicles* of transmission are infected cow's milk, infected cow's or pig's meat and infected sputum.

Although the gastric juice does not kill the Mycobacterium tuberculosis, still infection of the stomach is very rare. The only two places from which the bacilli may enter the body are (a) the bucco-pharyngeal and (b) the intestinal, mucous membranes.

Bucco-pharyngeal mucous membrane—The chief sites of entry are the tonsils, pharynx, lingual lymph follicles and the teeth sockets. The bacilli pass through the lymphatic circulation into the upper cervical and the submental glands. Though the infection is most commonly obtained from infected milk, the possibility of the above lesions in cases of pulmonary tuberculosis should also be borne in mind, as during coughing, the infected sputum scatters the bacilli all over these surfaces. The most important factor which facilitates the entrance of the bacilli through this channel is the unhealthy and chronically inflamed condition of the tonsils. Under such circumstances, the normal protective powers of the pharyngeal mucous membrane and the tonsils disappear and hence, the bacilli can easily pass through them. The rôle of pyorrhœa and carious teeth in transmitting a tuberculous infection should always be borne in mind.

The risk of a tuberculous infection through these sites by ingestion of milk is not very great in our country, owing to the fact that bovine tuberculosis is not so common here.

Intestinal mucous membrane—The bacilli penetrate chiefly through the lymphoid follicles and the Peyer's patches. Hence, the lesions are always more numerous and increasingly extensive as one goes towards the ileo-cœcal junction. *The bacilli can also pass through the mucous membrane without producing any gross lesion at the site of entrance.*

After penetration, they may develop the following lesions:—

(i) They may start an irritation and inflammatory reaction in the submucosa, specially in the lymphoid follicles which may soften and break down, producing *intestinal ulcers.*

(ii) Without producing any gross lesion, either at the site of entrance or in the submucosa, they may pass on to the nearest group of lymphatic glands, where they may be caught and where a tuberculous inflammation starts. In this way, glands after glands may be affected, producing the condition commonly known as *tabes mesenterica* (Fig. 170).

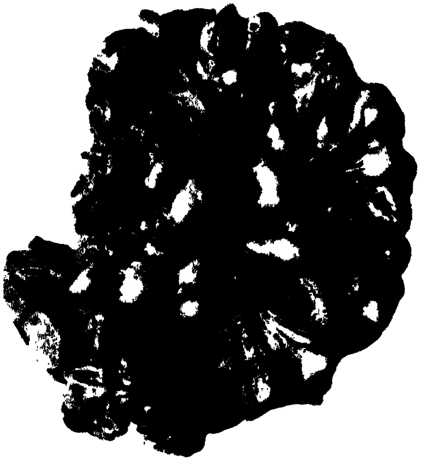


Fig. 170.
Enlargement of *mesenteric lymph nodes* **due, to tuberculous lesion (***Tabes mesenterica***).**

(iii) From the lymphatic glands, the bacilli may ultimately reach the general circulation, producing lesions in the lungs and other organs, or a condition of *general miliary tuberculosis* may ensue.

(iv) These glandular masses, by a process of contiguity or by rupture, may infect the general peritoneal cavity, producing a clinical picture of *tuberculous peritonitis.*

This mode of infection through the lower alimentary tracts is considerably facilitated, like that in the case of the bucco-pharyngeal mucous membrane, by any condition which produces a catarrh of the alimentary mucous membranes. Some of these conditions are chronic diarrhœa, measles and rickets. This last named condition produces a chronic catarrhal inflammation of all the mucous surfaces of the body, and hence, it acts as a very important predisposing factor to infection by Mycobacterium tuberculosis.

N.B.—Tuberculous ulcerations of the large bowel are not so common as those of the small gut. In the cœcum, however, a productive type of inflammation develops, which is often mistaken for a neoplastic mass,

Calmette's theory of tuberculous infection:—According to him, thoracic tuberculosis in adults and children is caused by bacilli which reach the lungs and the tracheo-bronchial lymphatic glands from the alimentary tracts by way of the lymph-and blood-stream, i.e., the infection is primarily lymphatic and then hæmal, and results chiefly from the absorption of the M. tuberculosis from the alimentary tracts. Although very strong experimental evidences are available from pathological and anatomical points of view to prove his theory of infection, there are other equally strong reasons to believe that this is not the usual mode of infection in cases of pulmonary and thoracic tuberculosis.

RESPIRATORY TRACTS: -In the majority thi the normal mode of infection in human tuberculosis, the disease being commonly transmitted by dried, finely divided sputum of phthisical patients.

Aschoff's theory of pulmonary tuberculosis:—According to him, human subjects get their **primary infection** in early childhood (within the first 5 years) by inhaling dried sputum which contaminates the dust and atmosphere. The bacilli lodge in some part of the lungs and produce a pulmonary lesion of varying degrees. This has been called by Aschoff as the "primary affect." After this, one or all of the following conditions may take place.

(1) The lesion, after reaching a certain stage, may involute up completely with fibrosis and calcification. Such a healed up l been referred to as the " Ghon's bodies " (*vide* Fig. 175).

(2) The lesion may increase and the infection may spread to the tracheo-bronchial lymphatic glands which may be considerably enlarged and caseous (Fig. 171). This condition of glandular enlargement may take place even

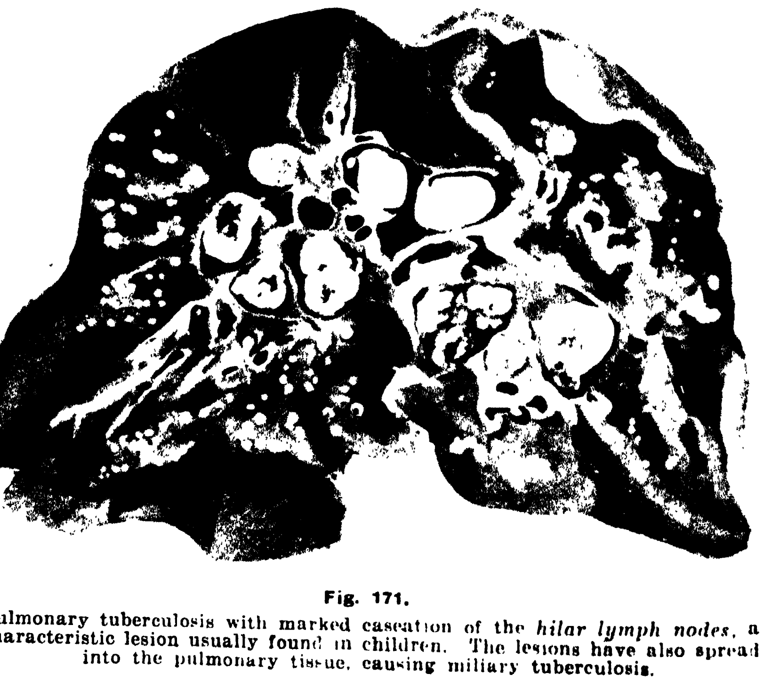


Fig. 171.

Pulmonary tuberculosis with marked caseation of the *hilar lymph nodes*, a characteristic lesion usually found in children. The lesions have also spread into the pulmonary tissue, causing miliary tuberculosis.

with a comparatively small pulmonary lesion. Such enlarged lymph nodes produce a characteristic shadow in X-ray pictures and are sometimes classified as "*hilus tuberculosis.*" Such cases, however, under suitable conditions, may also progress favourably and the lesions may heal up completely, leaving behind healed, calcified nodules in the lungs and calcified lymph nodes at the hilus (Ghon's bodies).

(3) On the contrary, the lesions may progressively increase in the lungs as well as in the neighbouring lymphatic glands, due to poor resistance and bad environments. Locally, the lung tissue is involved, leading to a wide-spread caseation and breaking down, producing a condition of caseous pneumonia or broncho-pneumonia (Fig. 172)--exudative type of lesion (*vide infra*). The infection may also pass to the general blood stream via the tracheo-bronchial lymph glands and the thoracic duct, producing a condition of generalised tuberculosis (acute miliary tuberculosis). These cases usually end fatally.

In those cases where the lesions involute and heal, the infection leaves behind two important effects in the host--*allergy* and *immunity*. The former is a condition of hypersensitiveness of the tissue cells to the tuberculous poison and on this depends the tuberculin reaction. The latter increases the resistance of the individual against subsequent infection.

Such persons in later life are constantly exposed to **re-infections,** but the course will vary according to the intensity and virulence of the infecting dose and the amount of immunity developed during the primary infection. For instance, *if the infecting dose is small and is received at sufficient intervals,* the immunity developed during primary infection may be enough to cope with it and prevent the

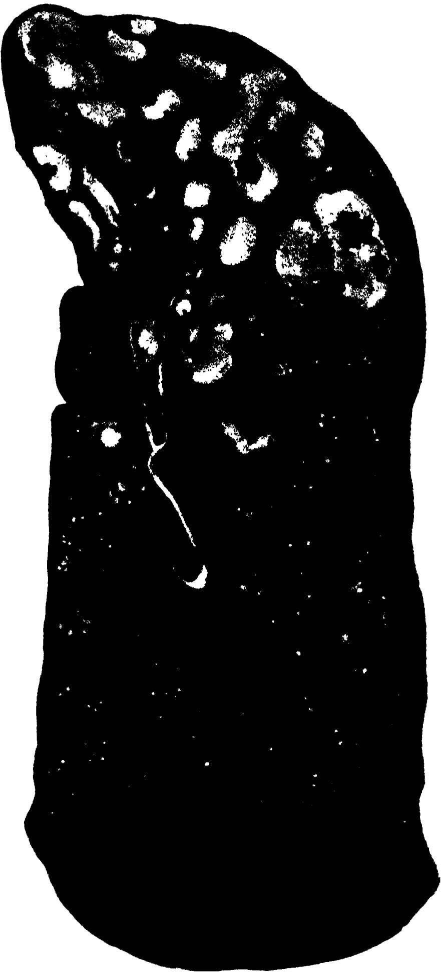


Fig. 172.

Caseous broncho-pneumonia (Phthisis florida; Galloping phthisis).

This is an example of exudative type of lesion which is usually found in persons with little or no immunity. Specimen obtained from a patient, aged 19 years, who suffered from signs and symptoms of acute pulmonary tuberculosis. Duration of illness was 2 months. Both the lungs were severely affected.

onset of the disease. Such a re-infection further increases the immunity to a greater extent. It is in this way, that the subsequent onset of tuberculous infection is prevented. This explains why human races who are never infected primarily with tuberculous infection in their early lives, offer a poor resistance when they are subsequently exposed to infection and usually suffer from a virulent type of the disease. In our country, the hill tribes from the Himalayas are typical examples. When they come down to the plains and become infected, they, being susceptible, invariably succumb very quickly to a rapidly spreading exudative type of the disease.

If the *infecting dose is heavy and massive, or is received in repeated small doses in quick succession* (this producing a cumulative effect), the primary immunity is unable to cope with the strength of the infecting dose and the result is that the bacilli gain the upper hand and start the disease.

Thus, in the opinion of Aschoff, cases of pulmonary tuberculosis in the adult life are always due to an exogenous re-infection by a massive dose of M. tuberculosis hominis.

Effects of the Mycobacterium tuberculosis on the tissues

The pathogenic effects are produced by endotoxins liberated on the death and disintegration of the organisms. This toxin is a very intense protoplasmic poison and, according to its intensity and concentration, may cause either (i) death of the tissue cells with *coagulative necrosis* or (ii) an irritation which stimulates *cellular proliferation*.

In this way, the tuberculous lesions are divided into two groups, the one, in which the degeneration and cell necrosis are more marked, is called *caseo-necrotic, destructive* or *exudative type*. This condition signifies that there is practically no immunity. It is particularly seen in young children and adults who had no primary infection in their childhood, e.g., the hill tribes. These cases usually run an acute course.

The other group in which cell proliferation is more marked is called the *formative, proliferative* or *productive type*. This condition signifies that there is a certain amount of immunity from a previous primary infection.

Usually, the above two effects are produced together, with preponderance of the one or the other, according to the amount and the intensity of the poison in any particular area and also to the resistance of the individual (Fig. 173).

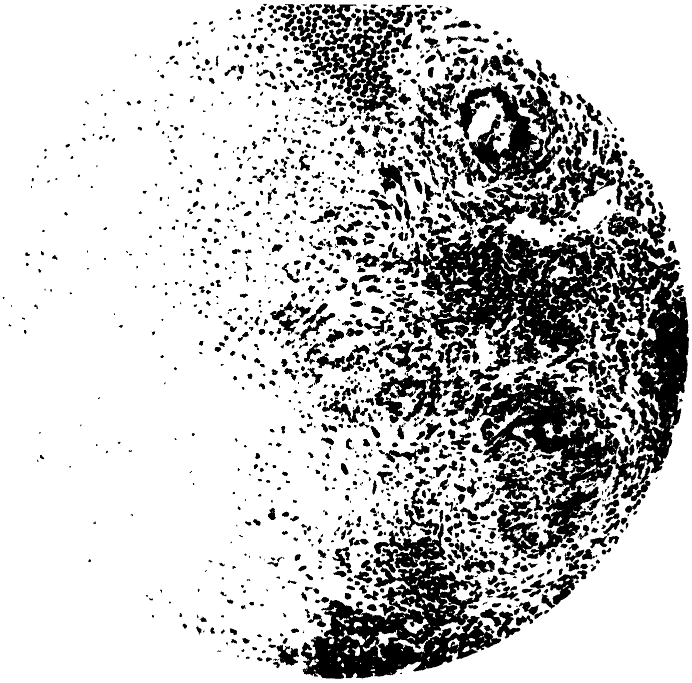


Fig. 173.
Photomicrograph of a tuberculous lesion, showing coagulative necrosis (caseation) and cellular proliferation.

The earliest visible manifestation of the reaction of the tissues to the invasion of the M. tuberculosis is the appearance of what is known as a " tubercle "- a minute, translucent, greyish white nodule, about the size of a millet seed. These "tubercles" are the pathognomonic lesions in tuberculosis.

Evolution of a " tubercle ":—The tuberculous toxin first of all damages some of the tissue cells, causing their coagulation necrosis and fatty degeneration. As this degenerated mass contains a large amount of fatty substance and looks like cheese, it is called caseation.

Along with this degenerative process, there is also irritation which results in the proliferation and accumulation of other cells which are derived partly from the connective tissues and partly from the blood, viz., the mononuclear, polymorphonuclear and the phagocytic wandering cells. Some of them die and add to the coagulative necrosis, but others make an effort to isolate the area. The cells which cluster round the degenerated central mass are usually attached to one another in a less orderly fashion to form a pale staining area. They have elongated vesicular nuclei with little chromatin and faintly outlined cell bodies which are irregular in form and sometimes branched, to connect themselves with their neighbours. These cells are commonly designated as the "*epithelioid*" or "*endothelioid*" cells by different observers. Some of these mononuclear cells fuse together, as if to work more harmoniously and to offer a more powerful combined resistance, thus losing their individuality and forming large plasmodial masses containing a large number of nuclei which are usually arranged around the periphery, forming the "*giant cells.*" At the margin of the "tubercle," there is a large accumulation of wandering cells and mononuclear leucocytes of the *lymphoid type.* The whole structure is supported by a newly grown connective tissue framework containing many reticulum fibres.

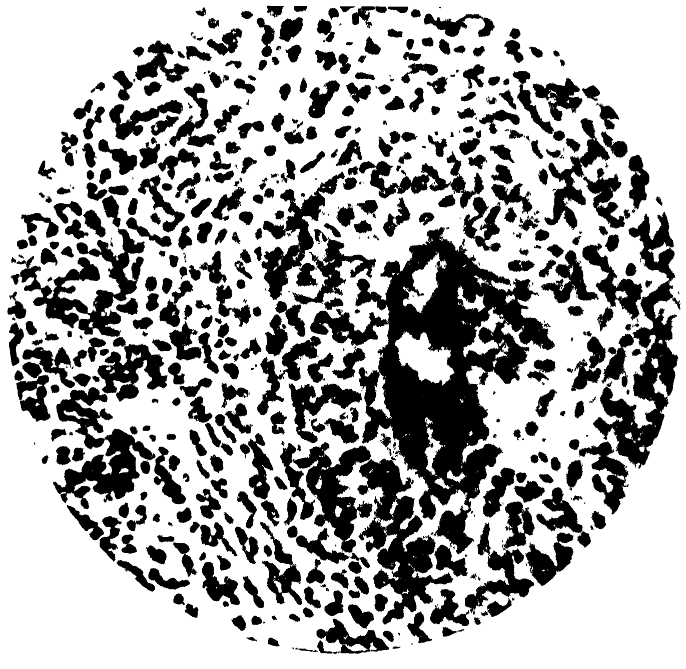


Fig. 174.

Photomicrograph of the *unit of lesion (tubercle)* in tuberculosis, showing a multinucleated plasmodial mass (giant cell) in the centre, surrounded by wandering mononuclear cells (endothelioid or epithelioid cells) which in turn are surrounded by lymphocytes. This is what is known as the "*giant cell system.*"

Such is the structure of a "*primordial tubercle*" (Fig. 174). The blood vessels at the point are obliterated due to thrombosis which also helps in the caseation. It would thus be seen that a "primordial tubercle" is an avascular element, but as it is very minute, it may absorb enough nourishment from the surrounding areas to keep it alive.

Modes of spread and the distribution of the lesions

Natural tuberculosis, to start with, is always a **local disease.** The primary focus may be in the lungs or in the intestines. *In adults, the characteristic tuberculous lesions are found mostly in the lungs.*

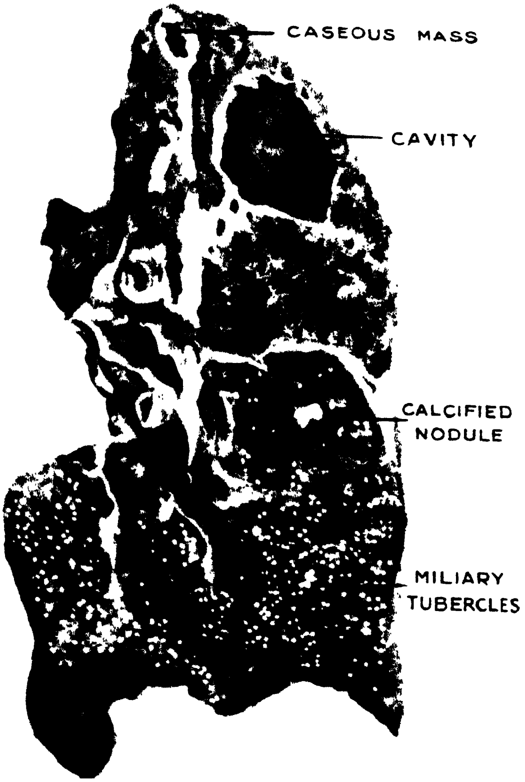


Fig. 175.

Gross specimen of a lung, showing the various types of lesions that may be found in pulmonary tuberculosis. This is an example of exudative-productive type of lesion. The calcified nodule represents Ghon's body or the healed lesion of the "primary affect" of Aschoff.

When virulent M. tuberculosis gains entrance into the body of a susceptible host, it initiates the tissue reactions characteristic of tuberculosis. As already stated, the subsequent spread of the lesion depends on the degree of immunity and the resistance to the tuberculous infection. Thus the lesion may spread locally or may serve as the origin of secondary tuberculous infiltrations in various other parts of the body.

In the *LUNGS*, wherever may be the starting point of the tuberculous process, a well defined lesion is formed. Sooner or later this undergoes caseation which may ultimately break down and open into an air tube through which the caseous mass is discharged with the sputum. Such a process leads to the formation of a cavity (vomica) which may progressively increase in size (Fig. 175).

In the case of the *INTESTINES*, a focus is formed in the submucous lymph follicle which may subsequently caseate and burst, forming an ulcer. At first, the ulcers are circular or oval, but later, they become ring-like (Fig 176). They rarely perforate but may, however, do so into another loop of intestine. The condition usually passes to the serous coat where the characteristic tuberculous deposits may be noticed along the lymphatic vessels (Fig. 177). When they undergo healing, they form characteristic cicatricial contractions (Fig. 178) which may produce intestinal obstruction.

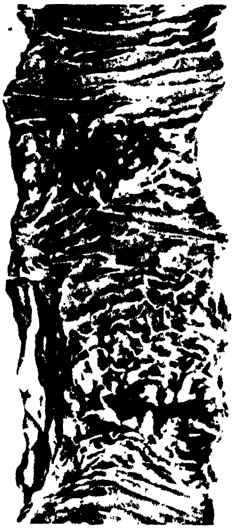


Fig. 176.
Tuberculous ulcer of the small intestine.

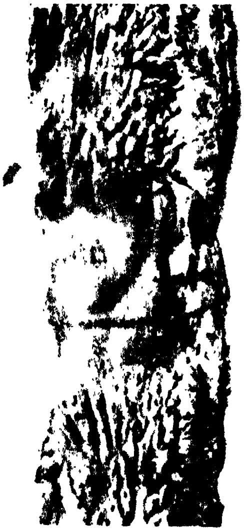


Fig. 177.
Peritoneal surface of the ulcer, showing tuberculous lymphangitis.

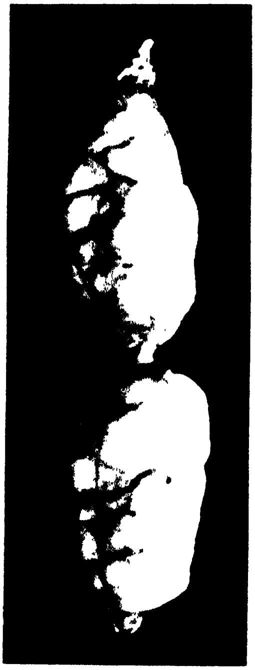


Fig. 178.
Stenosis of the small intestine, produced by healing of a tuberculous ulcer

The subsequent spread of the lesion from the primary focus takes place generally in one or other of the following ways:—(i) *by the lymph stream*, (ii) *by the blood stream* and (iii) *by a process of continuity and contiguity*.

1. *By the lymph stream*—This is very common in infants and children. The bacilli are transported, either free or inside the phagocytic wandering cells, through the lymph channels to the regional lymph nodes which become infected, enlarged and caseated. During such a transport, some of the bacilli

may penetrate the wall of the lymph vessels and cause characteristic lesions, e.g., tuberculous lymphangitis, often seen in tuberculous ulcers of the intestines. From these infected nodes, other sets of lymph nodes are affected and this process continues, until the bacilli find their entrance into the blood stream, via usually the thoracic duct. Then they are scattered all over the body through the blood stream, producing the condition known as *miliary tuberculosis*.

2. *By the blood stream*– The bacilli may gain entrance into the blood stream by the following ways:—

(a) Through the thoracic duct, as mentioned above.

(b) By rupture of a caseous focus and the subsequent discharge of the tuberculous material into a blood vessel.

When a large number of bacilli is disseminated, *acute miliary tuberculosis* develops without or with localisation in the lungs (Fig. 179), brain or abdomen. If, however, the number of bacilli is small, more chronic forms

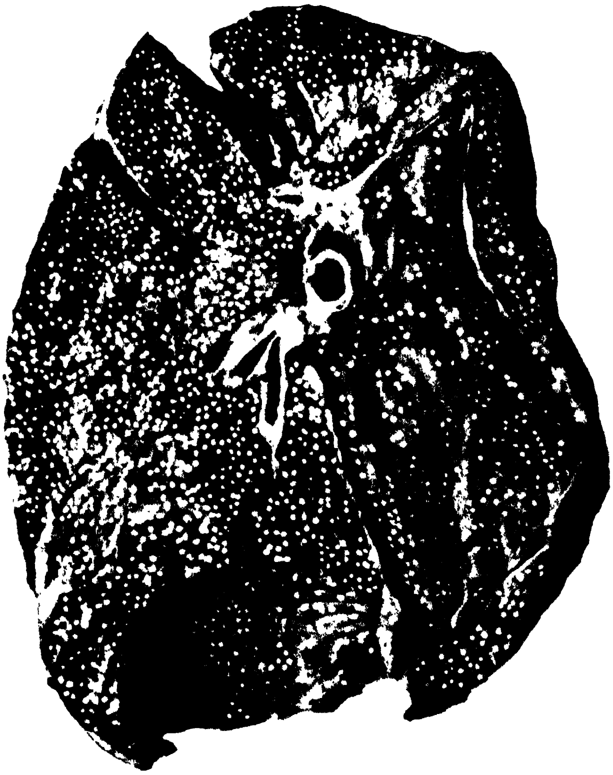


Fig. 179.

Acute miliary tuberculosis of the lung.
The greyish-white nodules are disseminated throughout the substance of the org

of tuberculosis are produced with localisation in the bones (Fig. 180), joints, kidneys (Fig. 181), genital organs, ductless glands (adrenals), etc.

3. *By a process of continuity and contiguity*—This mode of spread takes place when the primary lesion softens and breaks down, so that the infected material may be discharged into the various parts of the particular organ in question. As for example, when a pulmonary focus breaks down into the respiratory passage, the infected sputum may be aspirated, giving rise to a tuberculous broncho-pneumonia. Due to constant coughing, as well as constant contact of the infected sputum with the mucous membrane of the nasopharynx and the upper respiratory tracts, ulcerations develop in the trachea, larynx (Fig. 182), pharynx, tonsils, nose and the skin of the face (Fig. 183). The infected sputum may also be swallowed by ignorant people, giving rise to ulceration of the intestines, anal fistulæ, lupus of the anus and buttocks.

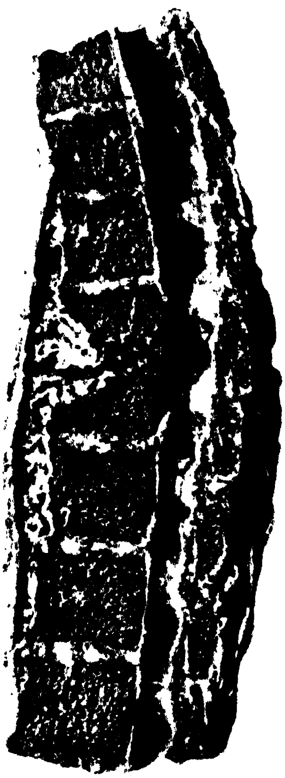


Fig. 180.

Tuberculous lesions of the vertebral column, showing the destruction of the interrtebral fibro-cartilaginous disc between the bodies of the sixth and seventh dorsal rtebræ. There is also cumulation of caseo aterial (cold abscess) l hind the anterior longit dinal ligament, projecting front and laterally to for a swelling.

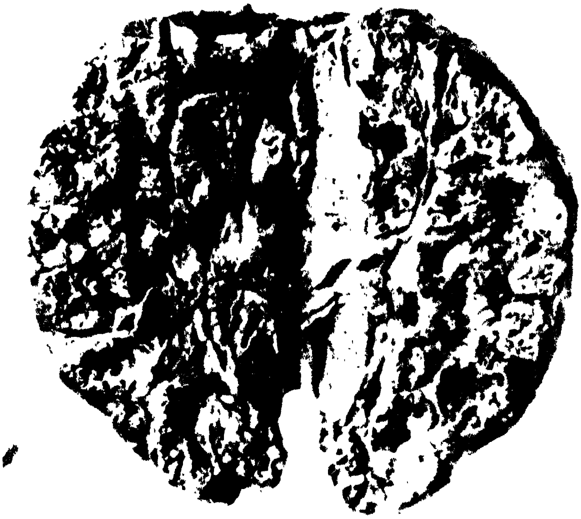


Fig. 181.

Tuberculosis of the kidney

Serous surfaces are often involved from an adjacent focus. Tuberculous peritonitis may develop from an intestinal ulcer, rupture of a caseous mesenteric lymph node, or from a diseased Fallopian tube (Fig. 184). Similarly, extension into the pleura and pericardium may take place from a pulmonary focus. In the same way, in tuberculosis of the kidney, the breaking down of the lesion will disseminate the bacilli into the lower urinary tracts and produce lesions in the urinary bladder, prostate and testis. This is known as the "descending infection."

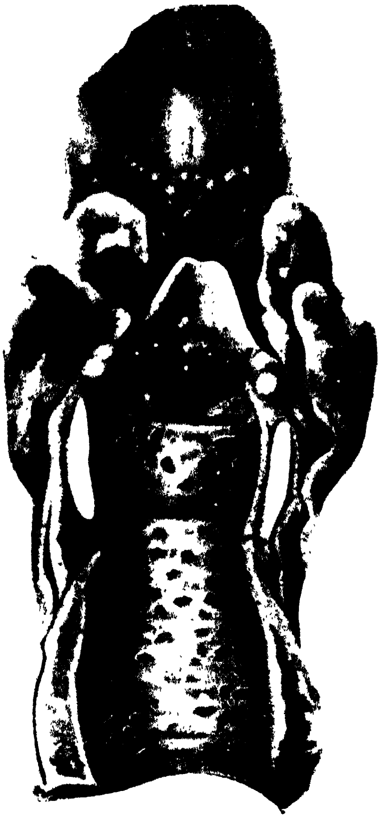


Fig. 182.
Tuberculous ulceration in the larynx and the trachea, secondary to pulmonary tuberculosis.

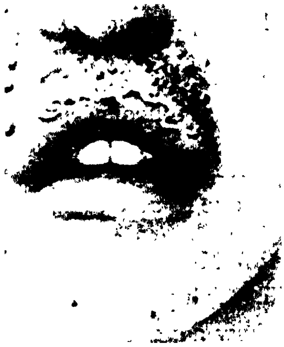


Fig. 183.
Lupus of the nose and lips. *(From Calmette's tuberculosis; by kind permission of Williams and Wilkins Company).*

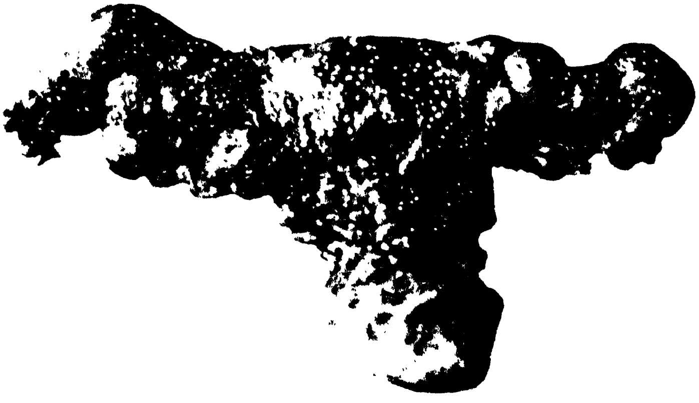


Fig. 184.
Miliary tuberculosis of the pelvic peritoneum in the female. The lesions have extended from the diseased Fallopian tubes.

Nature of the lesions in infants and young children

In these subjects, the disease always tends to generalise and involve the lymphatic system. Due to generalisation, miliary tuberculosis and tuberculous meningitis develop, usually terminating in death. Due to lymphatic involvement, there is an enlargement and caseation of the lymphatic glands. Such a change may take place in the following areas:—

(i) Tracheo-bronchial group—This is always secondary to a focus in the lung, produced by inhalation of the bacilli.

(ii) Cervical glands—The bacilli gain entrance through the bucco-pharyngeal mucous membrane.

Note.—In the adults, particularly in our country, such a condition is mostly due to an extension of the lesions from the lungs and the mediastinum.

(iii) Mesenteric glands—This is usually the result of ingestion of the bacilli which pass through the intestinal mucous membrane. The glands may also show advanced caseation, with or without intestinal lesions.

As the age of the child advances, the tuberculous process may be arrested in the glands or it may manifest itself in the various chronic forms of tuberculosis with localisation in the bones, joints, skin, kidneys or genital organs.

The part played by human and bovine types of M. tuberculosis in the causation of human tuberculosis:—

(1) Human tuberculosis may be caused by infection with either type of bacilli.

(2) Modern trend of opinion is that the human type of M. tuberculosis is the more frequent cause of human tuberculosis and that it is particularly so with the adult.

(3) The incidence of infection by the bovine strain, as a cause of human tuberculosis, is not very high in our country. It is an important source of human tuberculosis in Great Britain and particularly so in Scotland.

(4) Bovine type of infection is highest in children under 5 years of age, after which there is a progressive decline. The portal of entry is invariably the alimentary tract and the source of infection is the infected cow's milk. Of the various clinical forms of the disease, lupus and lymph-adenitis have yielded the highest proportion of bovine infections.

Mechanism of the production of symptoms of the disease

These are three:—

(1) **General effects**—The endotoxin and the products of metabolism of the M. tuberculosis produce fever, loss of weight, and anæmia.

(2) **Local effects** of the pathological lesions, by introducing abnormalities, interfere with the physiological activities of the vital organs. This depends on the organ affected. In the case of the lungs, there are cough, expectoration, haemoptysis, dyspnœa and pain in the chest (pleurisy). In the case of the intestines, there is diarrhœa with the passage of blood and mucus in the stool, with pain in the abdomen, ascites, obstruction, tumour formation, etc. The involvement of the mesenteric lymph nodes and the lymphatics leads to indigestion, fatty diarrhœa, etc.

(3) **Secondary effects** of the invasion by other micro-organisms—High; hectic fever, with or without chill and rigor, foul breath, abscess and gangrene of the lungs and pneumonia.

Relation between immunity and tuberculosis

In infants and children, the full mechanism for the protection of the body and life is not mature and consequently the resistance is feeble. Owing to this reason, even small doses of the infecting agent are capable of producing serious lesions. On the contrary, if the infecting dose is sub-minimal, and the child's resistance is fair, the bacilli produce only a localised lesion which ultimately heals either partially or completely, and which may or may not leave indelible marks. Any way, the child develops, in this way, some immunity against tuberculous infection to which he may be subsequently subjected.

It is for this reason that in our usual mode of life, although we are all frequently subjected to the risk of infection, the bacilli cannot get a firm foothold and start the disease as soon as they get into our system. Because, they find the tissues and the blood immunised to a variable extent and hence, they either die out or become innocuous. If, by some means, this immunity is broken, the body is undefended against the bacilli which then cause the infection. Some common conditions which often upset this immunity mechanism may be the following:—

Local—Trauma and strain, which are particularly responsible for bone tuberculosis.

General—(i) Conditions which lower [illegible] vitality, e.g., (a) certain constitutional diseases (Diabetes, chronic [illegible], kala-azar and chronic splenomegalic conditions, cirrhosis of the [illegible] dysentery, etc.), (b) alcoholism, (c) pregnancy and repeated [illegible] and (d) rickets in children.

(ii) Some infectious diseases which lower the general defensive mechanism of the body and give the bacilli a chance, e.g., measles and whooping cough in children, and influenza, typhoid fever and pneumonia in adults.

(iii) Certain environmental conditions, e.g., living in crowded, ill-lit and ill-ventilated houses.

(iv) Economic conditions, such as poverty and stress and strain of modern life.

THERAPEUTIC CONSIDERATIONS

Serum therapy:—Although antisera have been prepared by Maragliano (1889) and Marmorek (1903), passive immunisation in tuberculosis, up to the present, has not been attended with any success.

Tuberculin therapy:—The most suitable cases for tuberculin therapy are those with involvement of the glands, bones, skin and genito-urinary tracts. In cases of pulmonary tuberculosis, it is not so beneficial. The preparations used are old tuberculin and bacillary emulsion.

Dosage and the method of dilution:—With Koch's new tuberculin (B.E.), the initial dose is 1/100,000th of a milligram; the maximum being about 1/10,000th or 1/20,000th of a milligram. The dose is repeated at an interval of 4 to 7 days and is increased judiciously. The dilutions of tuberculin are carried out with 0·5 per cent. carbol-saline or sterile distilled water. The treatment is, however, continued for a long period.

How tuberculin acts:—Tuberculin treatment constitutes an example of active immunisation in which the patient's resistance to the tuberculous infection is raised by gradually increasing the doses of the toxin. The injected tuberculin, no doubt, leads to the development of antibodies (opsonic immune bodies and others), but the real benefit that ensues from its use is the production of the characteristic local reaction (allergic reaction) which helps in the encapsulation and healing of the diseased area.

Points to remember in tuberculin treatment:—It is very difficult to assess the value of the therapeutic results obtained by specific treatment with tuberculin. It is the firm belief of many clinicians that beneficial results are derived from such a treatment, but there is no doubt that overdosage may lead to definitely harmful effects. Favourable results appear to depend on a low grade focal reaction and it is quite likely that this limit may be exceeded to a harmful extent. Thus, this mode of treatment is not free from danger. Hence, tuberculin can be compared to a double-edged sword. If used for therapeutic purposes, the initial dose should be very small and should not excite any vigorous reaction. The subsequent injections should be made in judiciously increasing doses and at suitable intervals. After several repeated injections, marked immunity is developed. Another point to be remembered in tuberculin treatment is that the infected portion of the body should be kept at rest as nearly as possible and in this way auto-inoculation from the diseased area is prevented.

Prophylactic vaccination against tuberculosis:—

Vaccination with B. C. G. strain—The word B. C. G. stands for Bacille Calmette-Guérin. Calmette and his co-workers make use of a living strain of bovine tubercle bacillus that has become attenuated as a result of repeated subcultures for several years (230 subcultures extending over a period of 13 years) on a medium of potato, cooked in ox-bile with 5 per cent. glycerin. The vaccination strain was originally a very virulent culture but lost its virulence after repeated subcultures on the special medium. Though avirulent, it retained, however, its antigenic power in the sense that inoculation excited the formation of antibodies demonstrable by complement fixation test. It was first tried on young calves which showed a remarkable development of immunity against a heavy infection by a very virulent strain. Encouraged by the excellent results on animals, the B. C. G. vaccination was started on human infants. Calmette recommends 3 doses, each of 10 mgm. of the living avirulent bacilli, by the mouth, on alternate days, within the first 10 days of life. It is given by the mouth, because in the newborn, the alimentary mucosa is highly permeable. To be effective, it should be carried out within the first 10 days after birth, before the infant has had time to contract infection naturally, the object being to confer protection at the most susceptible period. It takes about a month for the immunity to be well developed.

Various objections have been raised as to the efficacy and safety of the procedure. They are as follows:—

(i) The use of a living attenuated culture involves the risk of the virulence being regained.

(ii) It is very difficult to standardise the dosage of living vaccines, as after the administration of the dose, the number of the organism may be increased inside the body.

(iii) Introduction of B. C. G. vaccine does not invariably give rise to allergy. If the condition of allergy is necessary for the development of immunity, then prophylactic immunisation with B. C. G. vaccines cannot be always regarded as a reliable method.

LABORATORY DIAGNOSIS OF TUBERCULOSIS

The final diagnosis of tuberculosis depends on the actual demonstration of the characteristic organism in the exudates and tissues. There is no other surer method than the above, but under certain circumstances, the direct methods may fail, when some other accessory methods are employed. Thus the methods of diagnosis fall under two heads—(i) direct and (ii) indirect.

A. Direct methods, i.e., establishment of a bacteriological diagnosis. This consists in the demonstration of the M. tuberculosis in excretions, discharges or tissues by (i) microscopic examination (Ziehl-Neelsen's stain), (ii) cultural examination and (iii) animal inoculation tests.

B. Indirect methods:—These are reactive phenomena produced as a result of the tuberculous infection. They are the following:—

(i) The presence of immune bodies in the blood (serological tests), which is determined by (a) complement fixation test and (b) determination of the tuberculo-opsonic index.

(ii) Phenomenon of hypersensitiveness or allergic manifestations (Tuberculin reactions).

(iii) Non-specific phenomena.

(a) Cyto-diagnosis of the exudate (preponderance of mononuclear cells).

(b) Tissue reaction, as found after histological examination of the diseased tissues.

A. Direct methods

1. **Microscopic examination**:- This is the simplest and the quickest method of establishing a diagnosis. A dried and fixed film of the specimen is stained by Ziehl-Neelsen's method and examined under the microscope (*vide* p. 292). Failure to detect the bacilli by this method is quite possible and this is particularly so when the organisms are very few in the specimen. In such cases, concentration methods, such as antiformin, ligroin or Petroff's method, may be adopted (*vide infra*).

2. **Cultivation**: This method is rarely resorted to for a routine diagnosis, as the growth of the organism takes a long time to develop. Isolation of the bacilli in pure cultures may be obtained, either directly on artificial media, or by injection into a susceptible animal, e.g., a guinea-pig, and then culturing from the lesions developed in the test animal. In the latter method, the material is usually taken from the lymphatic gland or the spleen of the animal and subsequently implanted on to a culture medium. This is by far the best and the surest method of obtaining pure cultures, but it always takes time.

Practical technique: –

(i) *If there is no secondary contamination*, any tuberculous material, such as a caseous mass, pus, pleural fluid or ascitic fluid, may be cultured directly on solid media, containing glycerol or albuminous substances. If the fluid is thin and the organisms scanty, it is better to take the centrifugalised deposit for inoculation.

In the case of tissues, the latter should be ground up in a sterile pestle and mortar, using sterile sand or broken glass. This should be well mixed with sterile normal saline which is decanted off and centrifugalised. The deposit may now be used for inoculation.

(ii) *If there is contamination with secondary micro-organisms,* inoculation may be made after the material is treated by one of the concentration methods (*vide infra*).

3. **Animal inoculation test (Pathogenicity test)**:—A sample in which microscopic examination has failed to reveal the presence of the causative organism, may be shown to be tuberculous in nature by this method. This test can also be utilised to prove the virulence of an organism suspected of being the M. tuberculosis.

For the purpose of carrying out this test, the animal of choice is the guinea-pig. Injection is generally given subcutaneously in the region of the thigh of a guinea-pig's hind limb, or the lower quadrant of the abdomen. Before inoculation, it is better to examine a smear stained by Gram's method and determine the amount of secondary infection. Specimens containing few or no secondary organisms may be inoculated without any preliminary treatment. If the material is heavily contaminated with secondary organisms, a preliminary treatment by antiformin or Petroff's method is advisable, otherwise the animal will develop an acute septicæmia (Streptococcal) before the evidence of tuberculous infection develops.

If the material contains M. tuberculosis, the guinea-pig generally dies within 6 weeks to 3 months, when it is a bovine strain, and within 3 to 9 months, if it is a human strain. Positive results of the disease may be suspected usually within 6 to 8 weeks, when the animal shows signs of the disease, e.g., gradual loss of weight from week to week. The animal may be killed at this time and a necropsy examination performed.

The *post-mortem* examination reveals the following:—

(i) A local caseous lesion at the site of the inoculation.

(ii) Enlargement and caseation of the proximal lymph glands (Inguinal).

(iii) A generalised tuberculosis, due to a rapid dissemination of the bacilli to other organs by the blood-stream. Tuberculous nodules of a yellowish grey colour are found in the spleen, liver and lungs (Fig. 185); the spleen being specially affected. The lesions in the inoculated animal are finally examined for the presence of acid-fast bacilli and for the characteristic histological picture.

NOTE:—Injections may also be given intra-peritoneally, provided there are not many secondary contaminating organisms, as the latter may kill the animal by producing peritonitis or general septicæmia.

CONCENTRATION METHODS

1. *Antiformin method*—Antiformin is a mixture of equal parts of liquor sodii chlorinatæ and a 15 per cent. solution of caustic soda. The former kills all other bacteria except M. tuberculosis which is resistant to its action

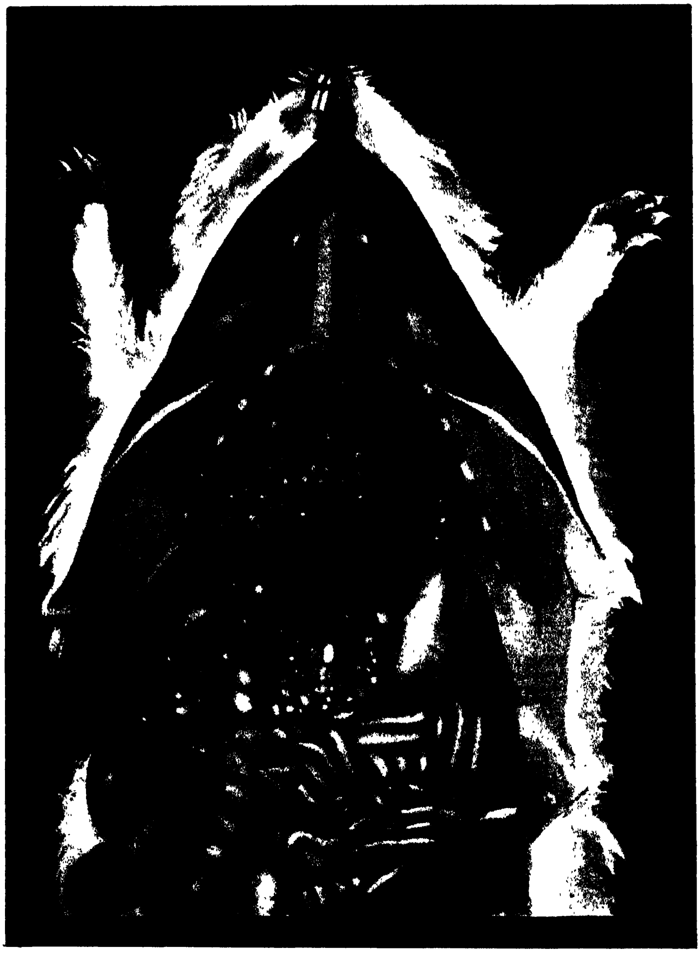


Fig. 185.

Post mortem appearance of a guinea-pig inoculated with a phthisical sputum, showing generalised tuberculous lesions. Caseous tubercles are seen in the lungs, the pericardium, the spleen, the liver and the lumbar lymph nodes.

and the latter helps to liquefy the mucus and assists the germicidal action of the former. Uhlenhuth (1909) first introduced the antiformin method. The technique has, however, been modified by A. S. Griffith (1916) who recommends a much simpler procedure. Equal parts (0·5 to 1 c.c.) of sputum and antiformin (10 to 30 p.c.) are mixed together and allowed to act for 10 minutes. With thin sputum, a 10 per cent. strength of antiformin should be used, but the percentages will vary according to the consistency of the sputum. It should be further noted that antiformin is a substance which must be used with caution, as a longer exposure will render the mixture sterile. In this method, the culture is to be made while the mixture is still mucinous and there is no necessity of centrifuging or neutralising.

The material may also be inoculated into the guinea-pig for pathogenicity test. For microscopic examination, it may be centrifuged and the smear made from the sediment.

In the case of the urine and fluid exudates, such as pleural, ascitic and spinal fluids, they are first centrifuged and the sediment thus obtained is mixed with a little distilled water and treated with antiformin. As these contain very little albuminous matter, it is advisable to add a little egg albumin or sterile serum, before adding antiformin or alkali. This also facilitates making of smears, otherwise they may not adhere to the glass slide.

With fæces, however, a heavy emulsion is made and then treated with antiformin in exactly the same way as described above.

2. *Petroff's method* (Technique):—Equal parts of sputum and a 3 per cent. caustic soda solution are well shaken and the mixture is incubated at 37°C. for half an hour. At the end of this time, the fluid is made neutral to litmus with dilute hydrochloric acid and then centrifuged. The deposit thus obtained is then planted on to the medium necessary for primary culture (*vide supra*). A pure culture of M. tuberculosis is thus obtained. Three per cent. caustic soda is strong enough to kill secondary organisms but has little or no effect on the M. tuberculosis. The deposit may also be examined microscopically after staining by Ziehl-Neelsen's method or injected into guinea-pigs to test for pathogenicity.

3. *Ligroin*, a petrol ether mixture, is also used by Lange and Nitsche (1909) for concentration purpose. In this method, the waxy tubercle bacilli adhere to the hydrocarbon and are carried up.

B. Indirect methods

1. **Presence of immune bodies in the blood (Serological tests) :—**

(a) *Complement fixation test*—This is usually carried on with M. tuberculosis or its product as antigen. Although this test indicates the presence of some antibody in the serum in a large number of cases, it is not infallible and may fail in some cases. It is owing to its uncertainty that the test has not gained popularity as a practical method of diagnosis.

(b) *Tuberculo-opsonic index*—The determination of the opsonic index is often considered as helpful in diagnosis. It has been found that this index falls down to a low figure in cases of tuberculosis. The value of the test as a diagnostic aid is not very great and hence its use is limited in practice.

2. **Phenomenon of hypersensitiveness or Allergic manifestations (Tuberculin reaction) :—**These reactions, as already described, denote a specific hypersensitiveness of the tissues to the [illegible]erculous toxin and are manifestations of allergy. The value of these [illegible]ns in the actual diagnosis of the disease is very great, specially in the [illegible] of young children of 2 to 3 years of age. With advancing age, how[illegible] the value of a positive reaction diminishes, as it may not indicate act[illegible]ase.

In early cases of **renal tuberculosis,** the bacilli are few and sometimes difficult to detect, the only finding being an acid urine with an occasional pus cell. To settle a bacteriological diagnosis, it is always advisable to use the animal inoculation test in such cases.

Detection of the M. tuberculosis in **fæces,** by microscopic methods, is often very difficult, but it has been greatly facilitated by the use of the antiformin and ligroin methods. Finding of the bacilli in fæces does not necessarily justify a diagnosis of tuberculous enteritis. The organism may be derived from the swallowed sputum and in children an examination of the fæces may sometimes be the only method of demonstrating a probable pulmonary lesion, since it is often difficult to obtain the expectoration in the case of a child.

Particular care must be taken in the microscopic examination of **urine and fæces,** owing to the possibility of the M. smegmatis being present. This bacillus is a harmless saprophyte which may be present in the external genitalia of both sexes. As already mentioned (p. 23), the M. smegmatis is acid-fast but not alcohol-fast. Hence, the acid-alcohol decolourising technique should be employed to overcome this confusion. To avoid such a contamination, it is always advisable to collect a catheter sample of urine.

Tissues, such as tuberculous glands, are sometimes sent for laboratory examination. A part of the tissue may be broken up by macerating it with sterile quartz or sand and emulsified with sterile saline. The supernatant fluid is pipetted off and treated with antiformin if necessary, and the subsequent examinations are carried on in the usual way (*vide supra*). The other part should be fixed and subjected to a histological examination.

TYPES OF MYCOBACTERIUM TUBERCULOSIS

As already mentioned, there are four main types of M. tuberculosis - human, bovine, avian (fowls) and piscine (fish).

M. tuberculosis bovis:—*Morphological differences,* however, are neither very marked nor very constant; although it has been remarked by some observers that the bovine bacilli are rather thick and short, their length rarely exceeding 1 micron, while the human bacilli are longer (about 3 microns), slender, often curved and more fragmented, after staining by the Ziehl-Neelsen method. Thus, it is not often possible to differentiate the bovine from the human types by morphology and staining reactions. The important differences lie in their cultural characters and pathogenic properties.

Cultural difference—The bovine type grows less luxuriantly than the human type and the presence of glycerin does not promote its growth. Thus, for the differentiation between the two types, culture may be made on Dorset's egg medium and glycerin egg medium. If the organism is M. tuberculosis bovis, the growth will be detected in the former medium but not in the latter one.

Virulence test—Mycobacterium tuberculosis bovis is very virulent for cattle and other domesticated animals. Of the laboratory animals, rabbit is the most important differentiating animal. An intravenous injection of 0·01 mgm. of a culture of M. tuberculosis bovis produces an acute general tuberculosis and death of the animal in 3 to 6 weeks. With M. tuberculosis hominis, however, the rabbit survives for *three months* or even more and on autopsy, the lesions are found to be slight and mostly confined to the lungs or kidneys or both. The other animals which may be used for differentiating purposes are calves in which the bovine type produces a generalised tuberculosis.

Table showing the differences between *Mycobacterium tuberculosis hominis* and *Mycobacterium tuberculosis bovis*.

	M. tuberculosis hominis.	**M. tuberculosis bovis.**
Cultural difference:—		
Facility to cultivate	Eugonic, i.e., easily cultivated.	Dysgonic, i.e., cultivated with difficulty.
Presence of glycerin in the medium	Assists growth.	Does not assist but inhibits the growth in earlier generation, but may assist later.
Nature of growth	Abundant, dry, verrucose growth; often yellowish.	Fine, white, powdery growth.
Viability in culture		Short.
	Virulent for man and monkeys. Guinea-pig is highly susceptible but less so than with the bovine.	Virulent for all species of mammals, including man. Highly susceptible animals are rabbits, guinea-pigs and calves.
Types of lesion in human beings:—		
Pulmonary tuberculosis ..	99 per cent	1 per cent.

Mycobacterium avium:—This is the causative organism of fowl tuberculosis. The morphological characters and the staining reactions correspond to those of the mammalian tubercle bacilli. The differences are, however, observed in cultural characters and animal inoculation experiments.

Its optimum temperature for growth is 40°C. at which the mammalian tubercle bacilli fail to grow. The bacillus can easily be cultivated and the cultures are more luxuriant, moister and readily form a homogeneous suspension.

Fowl is highly susceptible to the M. tuberculosis of avian origin, but is resistant to the human or bovine types. Of the mammalian species, rabbits and mice are susceptible to the avian bacilli. Human infections with the Mycobacterium avium are rather rare.

Mycobacterium piscium:—In morphological characters and sta reactions, it resembles other varieties of tubercle bacilli. Growth does not take place at the body temperature. It is probably a saprophyte, being occasionally associated with the disease in fish.

Mycobacterium paratuberculosis (Johne's bacillus)

This is the causative organism of chronic enteritis in sheep and cattle, producing nodular lesions in the small intestine. In morphology and staining characters, it resembles M. tuberculosis very closely. For cultivation, it requires the presence of other acid-fast organisms, preferably Timothy grass bacilli, which are added in the proportion of ½ to 1 per cent. dried extract to the egg medium. Like tuberculin, extracts of Johne's bacillus are prepared from cultures which are called *Johnin* and are found to be very valuable as specific diagnostic agents.

Organisms producing tubercle-like lesions in animals

1. **Corynebacterium ovis** (B. pseudotuberculosis ovis; Preisz-Nocard bacillus)—A natural pathogen of sheep, causing enlargement and caseation of lymph nodes and also caseous nodules in the internal organs. The organism is closely allied to C. diphtheriæ and resembles all its characters. In the experimental pathogenesis, however, it does neither produce any hæmorrhage in the adrenals nor any effusion in the pleural cavities.

2. **Corynebacterium murium** (B. pseudotuberculosis murium)—A natural pathogen of mice. It is also closely allied to C. diphtheriæ. So far as is known, it appears to be pathogenic only for mice and not for any other species.

Note:—The above two organisms combine the property of producing an infective granuloma, as well as a bacterial toxæmia.

3. **Pasteurella pseudotuberculosis** (B. pseudotuberculosis rodentium)—A natural pathogen for rodents, specially guinea-pigs. For the identification and differentiation of the organism, see under Pasteurella.

MYCOBACTERIUM LEPRÆ

(Bacillus lepræ)

The organism of human leprosy.

M. lepræ was first discovered in 1874 by a Norwegian bacteriologist, named *G. A. Hansen.* To commemorate his memory, we sometimes call the organism, Hansen's bacillus.

HABITAT:—It is not normally found in the human body; but in persons suffering from leprosy, the organisms are found in very large numbers in the nasal secretions, discharges from breaking down nodules and abundantly in the affected tissues.

MORPHOLOGY:—It is a slender, rod-shaped bacillus, of about 4 μ in length, resembling somewhat the Mycobacterium tuberculosis. The organisms are usually found together in large numbers, arranged like a bundle of cigars, inside the *lepra cells (vide infra).* The bacilli are bound together by a mucoid material called *glœa.*

The organism is *non-motile, non-flagellated, non-sporing* and *non-capsulated.* But, like the Mycobacterium tuberculosis, these bacilli are enveloped by a lipoid sheath around their bodies.

STAINING REACTIONS:—

1. Stained by the Gram's method, they are always Gram-positive.

2. Stained by the Ziehl-Neelsen's method, the bacilli are always acid-fast and take a solid and uniform staining. They are, however, unlike the Mycobacterium tuberculosis, less alcohol-fast, and sufficient exposure to alcohol will decolourise them completely. This fact should always be remembered, specially when demonstrating the organisms in sections of tissue. On such occasions, as little alcohol as possible should be used to dehydrate the section, as otherwise many of the stained bacilli will lose their colour.

CULTIVATION:—From the time of Hansen up till the present day, no reliable method has been available by means of which these Mycobacteria can be cultivated in artificial media. Many attempts have been made from time to time, but none of them has met with any success.

VIABILITY:—In the absence of our knowledge regarding its growth in artificial media, it is not possible to study the vitality and resistance of the Mycobacterium lepræ. From what could be known from indirect evidence, the bacilli may remain alive outside the human body for a considerable period, and may be capable of communicating the disease. This fact should be borne in mind when dealing with lepers and the articles used and infected by them. There have been definite and [illegible] instances where the disease has been contracted by initimate associ[illegible]ith lepers, as well as by using materials contaminated by them, quite [illegible] me afterwards.

TOXIN PRODUCTION:—No toxic product has yet been isolated from Mycobacterium lepræ. A patient may have an extensive and progressive cutaneous lesion, containing innumerable numbers of the organism, yet he does not show any toxæmic manifestation. In " lepra reaction," however, toxic manifestations are seen, but it is doubtful whether they are due to any true bacterial toxin.

SEROLOGICAL REACTION:—The serum changes in leprosy have been studied to a very limited extent. A complement fixation test and an increased sedimentation rate of erythrocytes have been described, but they are of very little use in the practical diagnosis of leprosy.

ANIMAL INOCULATION:—Up till now, no susceptible laboratory animal has been discovered for this kind of Mycobacterium. In this connection, it should be remembered that some animals, such as rats, naturally suffer from a disease which is called rat leprosy and which is due to an acid-fast organism, known as M. lepræ murium. Unless this is kept in mind, a false notion may be obtained when studying the inoculation tests for lepra bacillus on rats.

Distinction from the Mycobacterium tuberculosis

As the two varieties of the Mycobacterium are very much alike in morphology and staining reactions, it is well to remember some of the distinguishing features between them. These may be studied from the following table:—

	M. lepræ.	**M. tuberculosis.**
Morphology	Appear together in large numbers. Arranged in clusters and bundles, like a packet of cigars.	Appear singly or together in very much smaller numbers.
Staining	Take a solid stain.	Take a beaded and granular stain.
	Readily decolourised by treating with alcohol.	Not decolourised by treating with alcohol.
Cultivation ...	Can not be cultivated *in vitro.*	Can be cultivated *in vitro.*
Animal inoculation	No susceptible laboratory animal, hence unsuccessful.	Guinea-pigs and rabbits are very susceptible animals and hence, inoculation is always successful.

PATHOGENICITY

In human beings, M. lepræ produces the disease leprosy (*lepros*—scaly, *lepein*—to peel off).

Method of transmission:—This is not definitely known, but it has been suggested by many authorities that the disease is spread by contagion. Leprosy, like tuberculosis, is not a hereditary disease, as the children born of leprous parents, when separated, do not contract the disease.

Sources of infection:—The infecting organisms are usually discharged from ulcerating nodules and abrasions on the skin. They are also found in the nasal discharge, as well as in the droplets of saliva, which are expelled from the mouth of the patients during shouting, coughing and singing. All the above excreta may also infect food, wearing apparels, and similar household furniture. Naturally, any intimate contact with such patients is liable to carry the infection and spread the disease from person to person.

Portals of entry:—Although the actual mode of infection has not been definitely known, it has been suggested that the possible routes of entrance of the infecting agent are the nasal and the naso-pharyngeal mucous membranes and the skin surface. After entering the body, these Mycobacteria may remain dormant for a considerable period, even extending over years. Subsequently, when an opportunity occurs, due to some factors which probably tend to lower the body resistance of the person so infected, the bacilli grow rapidly and produce their pathogenic effects. The various factors which lead to the development of the disease are the following:—

(i) Intimate and prolonged contact.

(ii) Association with an infective case.

(iii) General resistance of the person exposed to infection. The last named factor is very important, as the organisms of leprosy will not usually grow in a healthy body. Anything which lowers the general resistance of the person will prepare the soil for their growth and act as a predisposing cause of the disease. These predisposing causes are more or less the same as mentioned under tuberculosis (pp. 313-314).

Pathogenic lesions:—The following are the common lesions produced by the bacilli in human beings only:—

1. Inflammation and ulceration of the ***nose and the nasal septum***—A sub-acute inflammation with a copious mucoid or mucopurulent discharge, occasionally tinged with blood, may be produced. Smear from such a nasal discharge shows a large number of the organisms, even in a very early stage before other lesions have become manifest. It is for this reason that some authorities consider this to be the initial lesion of the disease. Its importance is very great as it serves a fruitful source for the spread of the disease. An enormous number of lepra bacilli is continually discharged by the patient and is scattered all over and as such a person is quite unsuspected, owing to the absence of other external manifestations of the disease, he becomes a serious menace to the society by acting as a carrier.

Note:—Unlike syphilis, bones of the nasal septum are never involved.

2. In the ***skin,*** the Mycobacterium lepræ usually produces two distinct types of lesions; the one is characterised by nodular, granulomatous growths in the skin and subcutaneous tissues, affecting particularly the area of the face, nose and ears; the other consists of patches of macular, discoloured eruptions, which show marked trophic changes, such as anæsthesia, loss of hair follicles and sweat glands. According to the predominance of the one or the other type of changes, these cases are clinically called the nodular or the anæsthetic type of leprosy.

In **nodular leprosy,** large numbers of subcutaneous nodules develop, principally in the face, forehead, ears and sometimes throughout the body, involving particularly the extensor surfaces (Fig. 186). They vary in size and frequently ulcerate and slowly cicatrise with the formation of marked scarring and depressions. In some cases, the deformity thus produced resembles very much the face of a lion and hence, it is called a *leonine face* (Fig. 187). In this connection, it should be noted that these nodular cases are very infective, as the bacteriological examination of such lesions always reveals the presence of large numbers of bacilli. When ulceration occurs, the organisms are disseminated with the discharge which thus becomes a source for the spread of the disease. It is for this reason that these cases should always be segregated, if possible into a leper asylum.

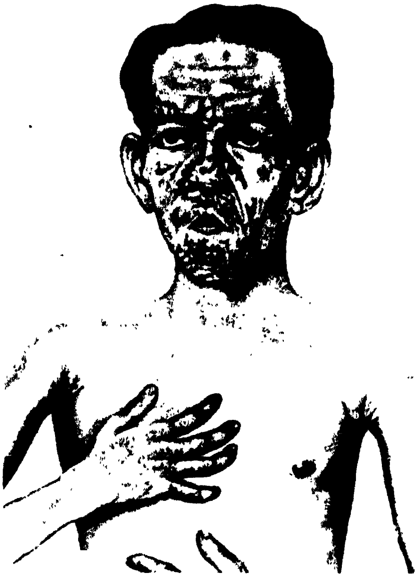


Fig. 186.

A case of nodular leprosy.

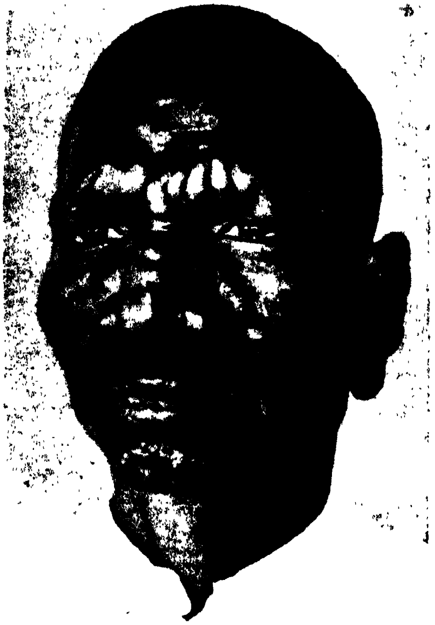


Fig. 187.

An advanced case of leprosy, showing the typical "leonine facies." Note the general thickening of the ear and face. (By kind permission of Dr. E. Muir.)

Morbid anatomy:—To the naked eye, the leprotic nodules, like any other inflammatory swelling, present, on section, a greyish, semi-transparent, uniform appearance. The loose, subcutaneous areolar tissues are affected and the yellow fatty appearance of the adipose layer is completely replaced by an avascular, whitish mass. On palpation, the nodules appear fairly firm and inelastic, and are found to end imperceptibly in the surrounding structures of the skin.

Microscopically, a section of such a nodule presents the picture of a specific infective granuloma. All the normal architecture of the skin and subcutaneous tissues is completely replaced by a chronic inflammatory exudate, consisting of all the cellular elements met with in a slowly cicatrising granulation tissue. There is a large number of fibroblasts and mononuclear cells. The latter are often vacuolated and epithelioid in type, containing the bacilli in their protoplasm. Besides, there may be seen many large vacuolated mono and multi, nucleated giant cells packed with the bacilli; these being usually known as the "lepra cells" (Fig. 188). Necrosis and marked caseation, as found in tuberculosis and syphilis, are wanting, as the nodules are comparatively better vascularised than the latter conditions.

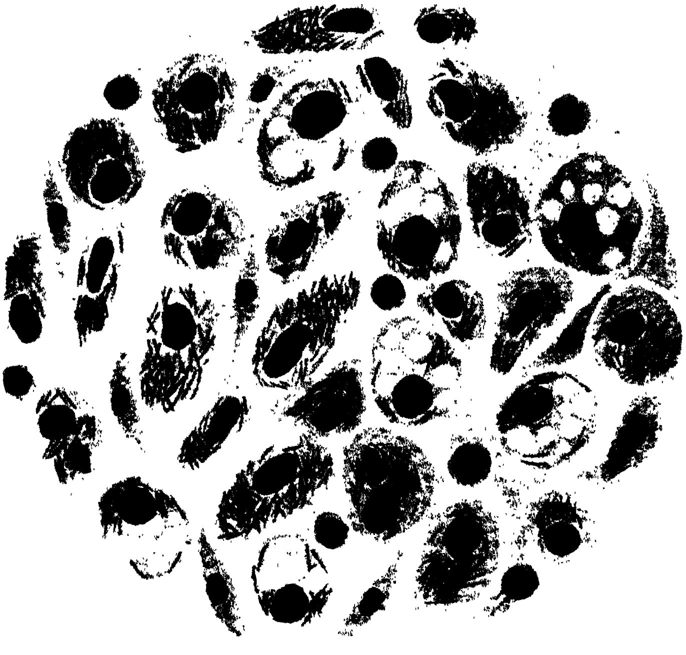


Fig. 188.

Camera lucida drawing of a microsection from a *lepra nodule*, showing the characteristic *lepra cells*. Stained by Ziehl-Neelsen's

A very characteristic feature of these nodules is that they are not painful and tender (presence of deep [illegible]sia) and the person does not complain of discomfort even at the time [illegible]ising such a nodule,

Lepra anæsthetica:—This is characterised by the appearance of depigmented patches in which one may find superficial anæsthesia (Fig. 189). The latter constitutes one of the very early signs. There may be patches of hyperæsthesia or anhydrosis (loss of sweat secretion), which are said to precede the formation of an anæsthetic patch. Such lesions are due to the involvement of the sensory nerve terminals in the skin and also of the non-medullated sympathetic nerve fibres. These patches, when examined bacteriologically, often yield a negative result and therefore are not infective. Hence, segregation is not essential in these cases. The disease tends to spread up the sensory nerve branches of the skin, involving the nerve trunks. The bacilli grow in the peri and endo, neurium where masses of granulation tissue, like those in the nodular types, are formed, producing fusiform

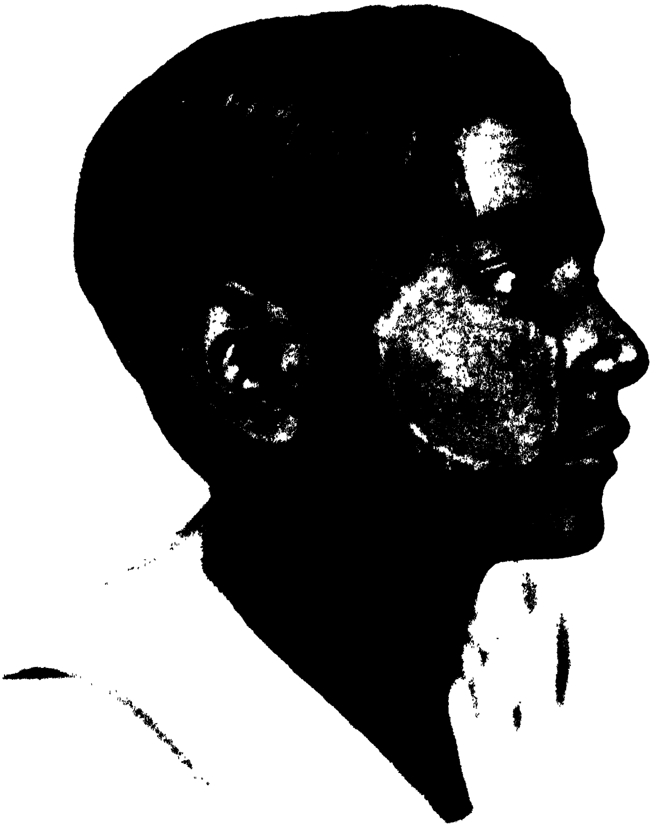


Fig. 189.

A case of *lepra anæsthetica*, an early lesion (A_1 case), showing the depigmented anæsthetic patch. Bacteriological examination was negative.

swellings in the course of the nerve trunks. The result is a neuritis, followed by hyperæmic patches and sensory, motor (muscular atrophy) and other trophic disturbances. Any of the superficial peripheral nerves may be involved, but the commonest ones, in the order of their importance, are the ulnar, the peroneal and the great auricular. The affected nerves are markedly thickened and, when they run superficially for some distance, they can be palpated as thickened cords. Finding of an anæsthetic patch with thickening of the corresponding nerve, is a strong presumptive evidence in favour of leprosy. It should be noted in this connection that these types of lesions are found in persons with a high resistance.

Atrophy of bones, specially of the small bones of the hands and feet, and ulcerations, including perforating ulcers in the soles of the feet, are examples of trophic disturbances, due to degenerative changes in the nerves. The organisms cannot, however, be demonstrated in such ulcers.

In both the varieties, the neighbouring lymph nodes always show some amount of enlargement, due to a mild chronic inflammatory reaction set up by the bacilli which reach them via the lymphatics. Mucous membranes are liable to be involved in this disease, producing inflammatory nodules, like those in the skin, although much smaller in size. The commonest examples of such lesions are found in the nasal, buccal and the conjunctival mucosæ. In the latter situation, there are produced iritis and phlyctenular conjunctivitis.

Course:—Leprosy is a self-healing disease and is not fatal by itself. It runs a very chronic course, being protracted for a number of years. The two types that have already been mentioned, depend on the amount of general resistance of the patient and at any time one may pass on to the other. To start with, most of the lesions are of the anæsthetic variety in which the bacilli are few in number. With the lowering of the general resistance, the bacilli increase in number and the nodular type of lesions is formed. These may progress on to ulceration and with the gradual development of immunity, may cicatrise or pass on to the secondary nerve cases (trophic changes).

It should be noted that the lepra cells act as hosts of the organisms which appear to live and multiply inside the cells. The presence of a lipoid envelope and a mucoid material (glœa) surrounding the bacilli protects them to a certain extent from the destructive action of the phagocytes. It is the disintegration of these bacteria-laden lepra cells and the liquefaction of the mucoid material that help the widespread dissemination of the organisms. During this period, the temperature rises and fresh lesions develop; this is what is known as the *reactionary phase of leprosy*. Any factor which lowers the general resistance will bring about such a reaction. Drugs, such as hydnocarpates and potassium iodide, which have the property of breaking down these lepra nodules, will also produce the same reaction.

From the descriptions given above, it will be seen that lepra bacilli always tend to get into the circulation and to produce lesions in the internal organs, such as the liver, spleen, lungs and testes, the last named organ being the commonest. The size and character of [illegible] lesions, however, never become so marked as in the case of syphilis and tuberculosis. Foci of infiltration with mononuclear cells and fibroblasts are produced and the bacilli are found inside the large, vacuolated "*lepra cells.*" In a very heavy infection, the bacilli may circulate in the peripheral blood in which they may be demonstrated, specially during a febrile paroxysm.

Though the peripheral nerves are affected, the central nervous system is never involved in this disease.

Classification of the lesions in leprosy (from Muir's leprosy):—The lesions in leprosy can be conveniently divided into 2 main groups as follows:—(1) Those in which the bacteriological examination of the skin, mucous membrane or the lymph nodes does not show the presence of the acid-fast bacilli. These are referred to as the A cases (nerve or neural leprosy) and are diagnosed clinically by the nerve signs, specially superficial anæsthesia and thickened nerves. As already mentioned, the fewer the bacilli in the body, the more is the disease confined to the peripheral nerves, signifying a greater natural resistance of the patient. The A type of cases may further be divided into A1 and A2, according as the case is a primary or secondary nerve one (the former showing patches and the latter trophic lesions).

(2) Those in which the bacteriological examination for acid-fast bacilli is positive are referred to as B cases (skin or cutaneous leprosy). This may again be divided into B1, B2 and B3, depending upon the increasing number of bacilli found.

Note:—Leprosy is a general disease and in no type are the lesions confined to a single tissue. The classification is, however, based on the predominant clinical findings.

THERAPEUTIC CONSIDERATIONS

Vaccine therapy has been recommended for the treatment of leprosy. For this purpose, suspensions of ground-up leprous nodules or of supposed cultures of leprosy organisms, such as Deycke's nastin and Rost's leprolin, are injected subcutaneously. It has been remarked by Muir that much benefit up to a certain point is often derived from these injections. The use of autolysed tubercle bacilli, as recommended by Row, has also been reported to give good results.

The most effective way of applying vaccine therapy is the administration of lepromalytes (hydnocarpates and potassium iodide), which causes the breaking down of lepra nodules and, in this way, acts as an internal autovaccination.

Various kinds of non-specific protein therapy have also been tried, the idea being to promote a general leucocytosis. Application of counter-irritants, such as painting of a solution of trichloracetic acid over the leprous lesion, produces a beneficial effect.

DIAGNOSIS

There are two cardinal points in the diagnosis of leprosy. The one is the presence of superficial anæsthesia in neural cases and the other is the finding of Mycobacterium lepræ in cutaneous cases.

Bacteriological examination:—This consists in the demonstration of the characteristic organism under the microscope by the direct examination of a smear stained by the Ziehl-Neelsen's method. The bacilli will be found stained red, in clumps and singly. As already mentioned, cultural examination and animal inoculation methods are of no use, except in the differentiation from M. tuberculosis. Thus, leprosy is one of the few diseases whose bacteriological diagnosis can be settled by morphological and staining characters alone.

Collection of materials:—

SKIN:—As the bacilli do not usually infect the epithelial cells, a superficial scarification is not enough and is, as a rule, useless. The material is, however, obtained by two principal methods.

(a) *Scraping method:*—The skin is cut vertically with a knife, about 2 millimeters deep, well into the dermis and the raw surfaces of the sides incised are scraped with the edge of a scalpel and smeared on a slide. When the bacilli are few in number, they cannot be found by this method.

(b) *Snip method:*—A small piece of the skin, at least 1 to 2 millimeters in thickness, is clipped off with a sharp pair of curved scissors. In a positive case, there is deep analgesia and little or no pain is caused. The cut surface has a white appearance and there is little or no bleeding. The cut surface of the excised piece is well rubbed on a clean slide or pressed on the slide so as to express as much as possible of the cellular elements. The bacilli will often be demonstrated by this method when they cannot be detected by the former method. If there is still any suspicion, the section should be subjected to a histological examination for the characteristic cell infiltration.

Note.—The best material for the above purpose is a subcutaneous nodule, but in its absence, the most convenient site to look for the bacilli is the edge of the *lobule of the ear* (Fig. 190). It is useless to take a clip from the superficially anaesthetic skin, as lepra bacilli are seldom found there.

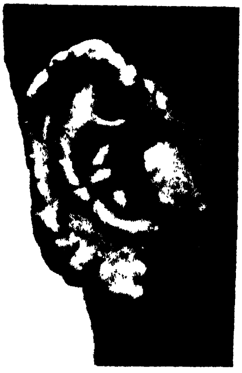


Fig. 190.

Leprous ear, the best place for taking a piece of skin for bacteriological examination. (By kind permission of Dr. E. Muir).

NASAL MUCOSA:—The next place from which material may be collected is the mucous membrane of the nasal septum. The anterior part of the septum is scraped with the point of a sharp knife, until a little blood begins to appear. The material on the edge of the knife is smeared on a slide and examined after fixing and staining. In cases of ulcerative condition, a swab may be taken from such a surface and the material stained in the usual way for the demonstration of the bacilli.

When there is an enlargement of the *LYMPH NODES*, a suitable one may be punctured and the juice obtained by applying suction, after injecting half per cent. cocaine and comminuting the contents of the node with the point of the needle. This is then examined in the usual way. Or the examination may be carried out by excising and sectioning the node.

In the matter of diagnosis, it should be borne in mind that the material discharged from the trophic lesions in the hands and feet and from the perforating ulcers in the sole of the foot is not suitable for diagnosis, as lepra organisms are not usually present in such areas. They are the results of trophic changes secondary to degeneration of the nerves.

Although the demonstration of the presence of the Mycobacterium lepræ constitutes the most reliable method of diagnosis, the latter are not obtained in many incipient cases, as well as in the early anaesthetic variety. Under such circumstances, the mucous membrane of the nasal septum should be carefully examined for any evidence of ulceration, and smears taken from the area and examined for acid-fast bacilli. A negative bacteriological finding should not be relied upon and the physician would do well to rely on his own clinical data.

Indirect methods:—

Besides the above direct methods, indirect evidences may be obtained from other sources, such as the *complement fixation test, blood sedimentation test* and *the effect of iodides.*

(i) *Complement fixation test*—A positive reaction may be obtained, but it has a very limited value in diagnosis.

(ii) *Erythrocyte sedimentation test*—In leprosy, the red cells sink much more rapidly than normal. The sinking speed of the red cells is observed in citrated blood. This test is not so important from the diagnostic point, but Muir has remarked that the test is invaluable for the prognosis and treatment of a case of leprosy.

(iii) *Effect of iodides*—As already mentioned, iodide is a substance which brings about reaction by breaking down the "ripe" lepra cells. In this way, it may be useful in determining the presence of unsuspected lesions in the skin, nerves, lymph nodes and the organs of the body. Potassium iodide is administered in 20 grains doses twice a week and increased up to 240 grains, the dose being doubled each time. Muir has remarked that in doubtful early cases, however, it is questionable whether it is the wisest course to try to elicit more definite signs in this way. He advocates that in these cases it is wiser to keep the signs suspicious of leprosy under observation and try to increase the patient's general resistance.

Leprosy and Dermal leishmanoid:—In our country, the latter condition is often mistaken for leprosy. Fig. 191 depicts the picture of a case of dermal leishmanoid which to many will appear and pass as leprosy, but a careful and detailed examination will reveal the true nature of the disease. The best way to settle the diagnosis in these cases is to clip a small piece from the nodule and make two smears from the cut surface. The one is stained with Leishman's and the other with Ziehl-Neelsen's stain. Microscopical examination of such stained smears will show either Leishmania donovani or Mycobacterium lepræ, as the case may be.

Note:—Rarely the two diseases may coexist in the same individual.

Leprosy and Syphilis:—These two diseases simulate each other very closely, as depigmentation and erythema are common in both of them, and in both, the corium of the skin is involved. It has been remarked by Muir that for the differentiation of the two diseases, Kahn's precipitation test is more reliable than Wassermann reaction.

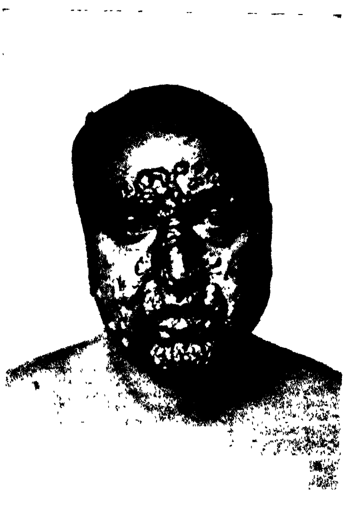


Fig. 191.
A case of *Dermal leishmanoid* which may be mistaken for a case of leprosy.

Leprosy and pulmonary tuberculosis:—Sometimes, when the disease involves the lungs, the patient may cough out, in his sputum, large numbers of M. lepræ. This may suggest that the patient is suffering from pulmonary tuberculosis. On the contrary, many lepers may get pulmonary tuberculosis as a complication and the finding of the acid-fast bacilli in his sputum may suggest pulmonary leprosy. Bacteriologically, it is sometimes not possible to decide the issue. For an accurate opinion, animal inoculation test should always be resorted to. In the case of leprous involvement of the lungs, no effect will be produced on the test animal, whereas in the case of a tuberculous infection of the lungs, the animal will show unmistakable signs of the disease.

Leucodermic patches and *ringworm affection of the skin* are often mistaken for leprosy. The absence of superficial anæsthesia and thickening of nerves in the above two conditions will settle the diagnosis.

Leprolin test:—This test is used to measure the degree of resistance to leprosy. In order to perform the test, two leprolins are employed. One is that of *Hansen's,* and which is prepared from leprous nodules, containing emulsion of M. lepræ and the other is that of *Stefansky's,* which acts as a control and is prepared from tissue lesions in rat leprosy, containing M. lepræ murium. In both the leprolins, however, some tissue debris is always present. The test is carried out by injecting [illegible] c.c. of each leprolin intradermally on the inner side of the upper arm. [illegible]itive reaction is characterised by the appearance of nodules at the sit[illegible] injection, the size of which is measured once a week for six weeks [illegible]rkedly positive reaction with

Hansen's leprolin, i.e., when the nodule is larger than the control, indicates increased resistance to leprosy.

Rat leprosy

This is a disease which is found in the domestic and ship rats, characterised by granulomatous affections of the skin, muscles and lymph nodes and caused by an acid-fast bacillus, the *Mycobacterium lepræ murium.* The causative organisms, which are found abundantly in the lesions, resemble the Mycobacterium lepræ in morphology and staining reactions. The cellular reaction and the histological picture of the lesions also resemble those of human leprosy; but, whereas the latter disease cannot be transmitted by experimental inoculation, rat leprosy is readily communicated from animal to animal by experimental inoculation methods. The other differences between human and rat leprosy are as follows:—

(i) Rat leprosy runs a more acute course.
(ii) Potassium iodide does not produce any reaction in rat leprosy.
(iii) Stroma of the muscles is invaded in rat, but not in human leprosy.
(iv) Nerve trunks and their branches are never involved in rat leprosy.

Other acid-fast bacilli

There are three main types of this group of organisms which are widely distributed in nature. All are non-pathogenic to human beings, leading a saprophytic existence. These are as follows:—(1) *Mycobacterium smegmatis,* found in the human smegma; (2) *Mycobacterium butyricum,* found in samples of butter, milk and cheese; and (3) *Mycobacterium phlæi* (Timothy-grass bacillus), found in grass and hay.

In morphology and staining reactions, these bacilli resemble very closely their more important pathogenic allies, but, whereas they are acid-fast, they are not at all alcohol-fast and they readily part with their fuchsinophil character when treated with alcohol. Another difference which these non-pathogenic Mycobacteria possess is their easy and rapid cultivation in ordinary media and at lower temperatures. For instance, they are readily grown, unlike the M. tuberculosis, in ordinary agar media at a temperature of 30°C. in 3 to 4 days, producing an abundant growth.

The importance of these organisms depends, however, on the fact that they may be confused with the Mycobacterium tuberculosis, during laboratory methods of investigations for the diagnosis of the latter. For instance, owing to their frequent presence in the human smegma, the M. smegmatis is a common contaminating organism in the urine. The finding of this acid-fast bacillus, during the bacteriological examination of the urine of a suspected case, may therefore suggest genito-urinary tuberculosis. Similarly, when milk is examined, the presence of M. butyricum may suggest tuberculous infection. The confusion becomes worse when we know that some of these non-pathogenic mycobacteria produce, when inoculated into guinea-pigs, lesions almost indistinguishable from those of tuberculosis. To avoid such confusion, it is desirable that absolute alcohol be used sufficiently during the time of staining by the acid-fast method. By this means, the non-pathogenic members of the mycobacterium group may be readily made to lose their acid-fast characters and thus safely excluded.

CHAPTER XXIV

CORYNEBACTERIUM

The characteristic of this species of bacteria is their tendency to the formation of club-like swellings at the poles (" Coryne " means 'a club').

Examples:—

A. Pathogenic to man and animals:—

1. *Corynebacterium diphtheriæ* (B. diphtheriæ)—The causative organism of diphtheria.
2. *Corynebacterium ovis* (B. pseudotuberculosis ovis; Priesz-Nocard bacillus)—The causative organism of ulcerative lymphangitis of horses and pseudotuberculosis of sheep.
3. *Corynebacterium murium* (B. pseudotuberculosis murium)—The causative organism of pseudotuberculosis of mice.

B. Non-pathogenic to man and animals (Diphtheroid group of organisms):—

4. *Corynebacterium hofmanni* (B. hofmanni).
5. *Corynebacterium xerosis* (B. xerosis).
6. *Corynebacterium acnes* (B. acne).

CORYNEBACTERIUM DIPHTHERIÆ

(Bacillus diphtheriœ)

HISTORICAL:—From clinical and pathological observations, *Bretonneau*, in 1826, regarded diphtheria as a specific disease and separated it into a clinical entity.

In 1883, *Klebs* observed the organisms in the false membrane and described their characters but made no cultivation.

It was *Loeffler* who, in 1884, first isolated the organism in pure culture from the throats of patients suffering from clinical diphtheria. He gave an account of its cultural characters and its pathogenic effects on animals. The organism is, for this reason, known as the *Klebs-Loeffler's bacillus* or simply as *Loeffler's bacillus*.

In the year 1888, *Roux* and *Yersin* [illegible] an important discovery that a potent extra-cellular toxin can be obta[illegible]m the filtrate of a culture of diphtheria bacillus. The lesions produ[illegible] this toxin were exactly identical with those which followed the inje[illegible] the living organisms. Thus it was established that C. diphtheriæ produced pathogenic lesions through its exotoxin and that there was no spread of the organisms beyond the local lesions.

In 1890, *von Behring* (Fig. 192) first studied the immunological aspects of C. diphtheriæ and was able to establish beyond doubt that a specific antitoxic serum could be prepared to neutralise its toxin and thus confer immunity against the disease.

Fig. 192.

Emil von Behring (1854 1917),
(By courtsey of Bayer Meister Lucius).

No further serious discovery was made after this till the year 1908, when *Bela Schick* of Vienna succeeded in introducing her specific method, the Schick test, in diagnosing the susceptibility of persons to diphtheria infection. On the basis of this specific test was started the active immunisation of susceptible children by a toxin-antitoxin mixture with marked success.

HABITAT:—The organisms do not normally inhabit the naso-pharyngeal mucous membrane but may be found in that situation in cases of carriers. They are always found in persons suffering from diphtheria.

MORPHOLOGY:—

Shape:—Slender rods, with a tendency to show clubbing at one or both ends.

Size:—3 to 4 μ in length by 0.3 to 0.5 μ in thickness.

Arrangement:—They usually lie in small clusters, parallel to one another or at various angles, so as to resemble the letter V or L or combinations of these, which have been compared to Chinese letters or cuneiform characters (Fig. 193). This peculiar arrangement appears to depend on the mode of division of the bacilli. During reproduction, when a bacillus divides into two, the cell membrane separates at one side completely but remains continuous at the other end for some time. Hence this acts as a sort of a hinge on which the bacilli can work and arrange themselves in L, or V shaped or any other angles. By a repetition of such a process among adjacent bacilli, the organisms are pushed together and the parallel grouping is thus assumed.

It is a *non-capsulated, non-sporing, non-flagellated* and *non-motile organism.*

Metachromatic granules *(Volutin granules or Babes-Ernst granules):* —When stained, the protoplasm of the bacillary body takes the stain unequally, giving a sort of barred or beaded appearance, due to specific intracellular granules. Owing to the presence of these granules, the bacilli give a metachromatic reaction with certain dyes and hence, they are referred to as " metachromatic granules " (*Meta,* after; *Chrome,* colour). They are usually situated at the poles of the bacilli and are known as 'polar bodies' or 'polar granules' (*Vide* Fig. 193). They may also occur in other parts of the bacterial body and the longer bacilli may contain several of them.

Polymorphism or Pleomorphism:—This is very characteristic of the species because of its wide variations in length, development of the terminal swelling and metachromatic granules.

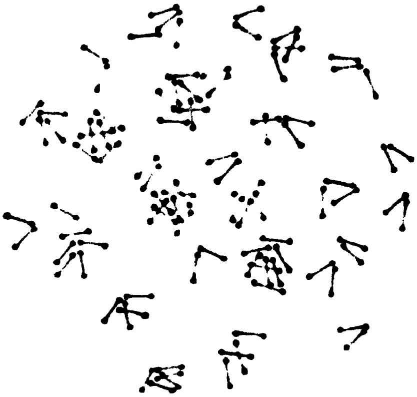


Fig. 193.

Morphology of *Corynebacterium diphtheriæ*, showing the characteristic Chinese-letter-like arrangement.

Involution forms (Fig. 194): —These forms represent the degenerated and distorted forms of the organisms, which appear when the cultures are incubated for more than 24 hours. This is due to old age and also to unfavourable environmental conditions, resulting from the exhaustion of the nutrient matter in older culture media. The bacilli become thicker, longer, segmented or curved. They may be pearshaped, club-shaped and globose.

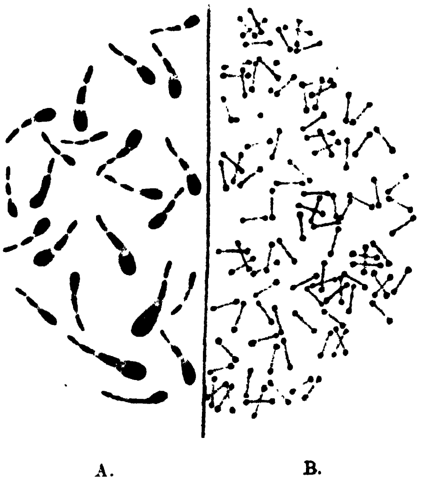


Fig. 194.

Involution forms (A) of *Corynebacterium diphtheriæ* as compared with the normal forms (B).

STAINING REACTIONS:—

1. ***Aniline dyes:***—They take up the basic aniline dyes with great readiness and have a special affinity for *alkaline methylene blue* (Loeffler's). With this stain the granules stain deeply and become prominent, giving the metachromatic reaction as described above. A beaded appearance is thus produced and the bacilli may be mistaken for streptococci.

2. ***Gram's stain:***—It is Gram-positive.

3. ***Special stains*** (see page 24):—With these stains the metachromatic granules are better demonstrated (Fig. 195).

Principle of the stains:—As the metachromatic granules are composed of substances allied to nucleo-pr[illegible], they are more deeply stained than the remainder of the bacterial prot[illegible] specially in an acid solution,

CULTURAL CHARACTERS:—

Biology:—The organisms grow best at a temperature of 37·5°C. in a medium containing serum or egg. They are aerobic but facultatively anaerobic.

Cultivation:—On good nutrient agar, the organisms may grow, but scantily and after prolonged incubation. *Colonies* are very small and resemble those of streptococci. They are small circular discs of an opaque creamy whitish colour. Better growth is obtained in glycerin agar (glycerin 2 to 3 per cent.) or serum agar or glucose agar.

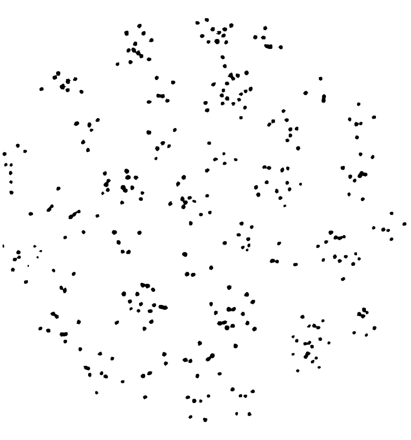


Fig. 195.
Metachromatic granules of *Corynebacterium diphtheriæ*. Stained with von Staltenberg stain.

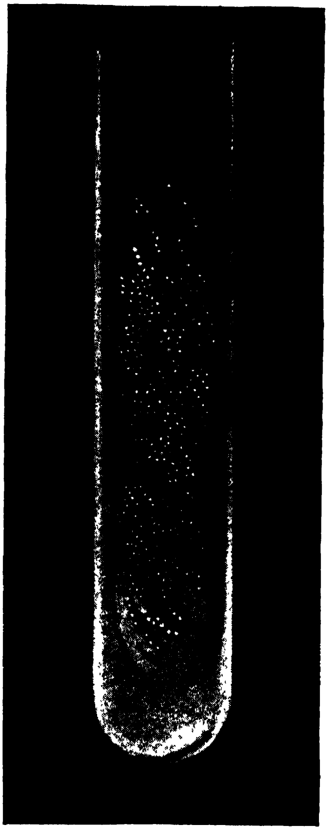


Fig. 196.
Growth of *Corynebacterium diphtheriæ* on solid medium (Loeffler's medium), showing the characteristic raised "poached-egg"-like colonies.

NOTE:—Ordinary nutrient agar is not suitable for the culture and diagnosis of C. diphtheriæ.

In **fluid media** such as glucose broth or serum broth, the organisms produce a granular deposit at the bottom with sometimes, a pellicle formation on the top.

Special media (p. 63) used for the purpose are Loeffler's serum medium, Dorset's egg medium and serum agar. The bacilli grow in Loeffler's medium within six hours and before other secondary organisms have had time to grow; for this reason, it is very suitable for primary cultivation and rapid diagnosis.

Character of the colonies—The colonies on solid media are discrete, opaque and cream coloured or pale yellow in colour. They are circular and convex, with a slightly raised centre, having a ' poached egg ' appearance (Fig. 196).

Tellurite ~~egg~~ medium (*vide* p. 64)—This is specially used for the isolation of the true diphtheria bacillus from the other non-pathogenic members of the Corynebacteria group. C. diphtheriæ produces black colonies with a greyish white periphery while the diphtheroid group produces greyish white colonies.

In order to prove the pathogenicity of a given strain of C. diphtheriæ, it is necessary to cultivate it in the following ways:—

1. *Primary culture on Loeffler's medium*—All primary inoculations from the infective material must be made on a medium rich in serum and for this, Loeffler's inspissated blood serum medium is the best.

2. *Secondary cultures on serum agar plates*—In order to obtain a pure culture, subculture may be made on serum agar plates. Selective medium containing telluric acid may also be used for this purpose.

3. *Cultivation of the pure organism in broth*—In order to prove that the organism produces a diffusible exotoxin, it is grown in a fluid medium, such as broth.

BIOCHEMICAL REACTION:—Fermentation reactions are best performed in Hiss's serum water medium (pp. 72 & 87). This is filtered horse serum, diluted 1 in 4 with distilled water, and sterilised by steaming at 100°C. for 3 successive days. Sugars, 1 per cent., and indicators may be added, either before or after. The organism produces *acid but no gas in glucose, maltose, galactose* and *dextrin*. It *never ferments saccharose* and *lactose*.

In *milk*, there is an abundant growth of the organisms with acid production but no clotting.

Gelatin is not liquefied.

VARIETIES OF CORYNEBACTERIUM DIPHTHERIÆ

M'leod and his co-workers have been able to distinguish two forms of C. diphtheriæ, chiefly according to their cultural characters. They are (i) *C. diphtheriæ mitis* and (ii) *C. diphtheriæ gravis*. The differences between the two are shown in the following table.

	C. DIPHTHERIÆ GRAVIS.	**C. DIPHTHERIÆ MITIS.**
Character of growth:—		
On solid media ...	Flattened lustreless colonies of irregular outline.	Colonies are convex and partly translucent.
In broth ...	Granular deposit at the bottom and pellicle formation on the surface.	Uniform turbidity.
Hæmolytic activity ...	Negative.	Positive.
Fermentation of polysaccharides (starch & glycogen) ...	Positive.	Negative.
Toxic phenomenon ...	Very marked.	General toxic symptoms tend to be slight, though there may be formation of an extensive false membrane.

NOTE:—In an epidemic, the virulent ca " due to the gravis type of bacilli. The
mitis type, although found to produce mild s in human infection, gives rise to pro-
fuse toxin formation in cultures and is eq ogenic to guinea-pigs. The strain
commonly known as the " *Park Williams No* f the nature of *C. diphtheriæ mitis.*

RESISTANCE:—Unlike other pathogenic bacteria, this organism can withstand desiccation and exposure to light much more considerably.

Drying—The bacilli, in the dried condition, can survive for a longer period. In a dried membrane, the organisms resist a temperature of 98°C. for one hour. When kept at room temperature and in a dark place, they may remain alive and virulent for several months. These facts should be remembered in considering the mode of spread of the disease.

Temperature—The organism, in the vegetative state, cannot resist heating very much and the thermal death point is 58°C. for 10 minutes (*Welch* and *Abbot*). Low temperatures and freezing are well borne.

Antiseptics—It is easily destroyed by chemical disinfectants in strengths customarily employed. Hydrogen peroxide seems specially efficacious in killing the organisms.

TOXIN PRODUCTION:—The very fact that the bacilli remain localised and are not disseminated throughout the body and still produce serious symptoms led *Loeffler* to infer that the organisms elaborated a poison which produced the symptoms after absorption. It was *Roux* and *Yersin* who first demonstrated the presence of an extra-cellular toxin (*exotoxin*) in the filtrate of a broth culture of C. diphtheriæ. This filtrate, when injected into a susceptible animal (guinea-pig), produces practically the same effect as the living bacilli.

The production of exotoxin varies with different strains. It has been found that a particular strain, known as "Park Williams No. 8," produces the maximum amount of exotoxin and hence, the same is used for the preparation of antitoxic sera.

The nature and various properties of exotoxin are already mentioned on p. 39 and p. 154. It will be seen further that in a filtrate of broth culture of C. diphtheriæ, 3 kinds of toxins may be found. They are as follows:—

(a) *Pure toxin,* where both the toxophore and haptophore groups are present.

(b) *Toxoid* or *anatoxin,* where the toxophore group has suffered deterioration but the haptophore group has not undergone any alteration, and hence, the latter has still the power to act as an antigen and to combine with antitoxin.

(c) *Toxone,* which possesses a toxophore group different from pure toxin and hence has a different toxic action. Its local action on tissues is very slight and it is responsible for the production of late nervous phenomena. It has also a weaker affinity for antitoxin.

SEROLOGICAL REACTIONS:—The following antibodies have been demonstrated in an immune serum.

(i) *Antitoxin*—This is the most important antibody and is largely used for therapeutic immunisation.

(ii) *Bacteriotropin* of Neufeld or thermostable opsonin which exerts a stimulating action on the phagocytes, *in vitro* as well as *in vivo.*

(iii) *Precipitin*—Its presence is demonstrated by the occurrence of a flocculent precipitate when an antiserum is mixed with a toxin or anatoxin. This reaction is now being employed for the standardisation of antitoxin.

Agglutinin, bacteriolysin and *complement-fixing body* are not usually found in antisera.

ANIMAL INOCULATION:—Of the various laboratory animals which are susceptible to diphtheria toxin, the *guinea-pig* is the most important and is very sensitive. Rabbits are less susceptible and the mice and rats are practically immune.

Animal inoculation is generally employed to test the virulence of an organism which has been isolated from the throat of an infected person or of a carrier. It is also used to test the potency of an exotoxin obtained by filtration.

The animal of choice is the ***guinea-pig*** and the method most commonly employed is the ***subcutaneous inoculation.*** A local lesion manifests itself within 12 hours and at the end of 24 hours, the animal shows definite signs of serious illness. It loses weight rapidly, the temperature falls and the animal becomes dyspnœic. The lowering of the temperature is due to the gradual failure of the vital powers. The animal usually dies within 4 days. It is always advisable to use two guinea-pigs for the test. One of the animals which serves as a control, is protected by an injection of 500 units of anti-diphtheritic serum, 12 to 24 hours before the test is carried out. If the organisms appear to be virulent, they will not produce any ill effects in the control animal, but the test animal will manifest all the signs of illness mentioned above and post mortem, the following lesions will be found.

Characteristic post mortem appearances (Fig. 107):—

Local effects—At the site of the inoculation, a massive, gelatinous and sanguineous œdema is developed. Usually, there is a varying amount of inflammatory exudate in the form of a thin greyish white membrane. The regional lymph nodes are enlarged and congested.

General effects—The most constant and characteristic lesion of experimental inoculation is found in the *adrenals*. Both the organs are enlarged, congested and dark red in colour. On section, extravasation of blood (hæmorrhages) is seen inside the substance of the organ. Besides the above, there is a marked congestion of the kidneys which appear bluish purple in colour, œdema of the lungs, effusion into the pleural and pericardial cavities and necrotic changes in the heart and liver.

Note:—C. diphtheriæ always remain strictly local and never disseminate into the general blood stream.

Intradermal test (*Okell* and *Parish*): In order to test the virulence of the organism, it is isolated in pure cultu[illegible] cultivating it on serum agar plates. After 18 hours' incubation, a sali[illegible]sion is made and standardised to contain 500 millions of organisms ; [illegible]. Two white guinea-pigs of standard weight are taken. One of them [illegible]ected by an intraperitoneal

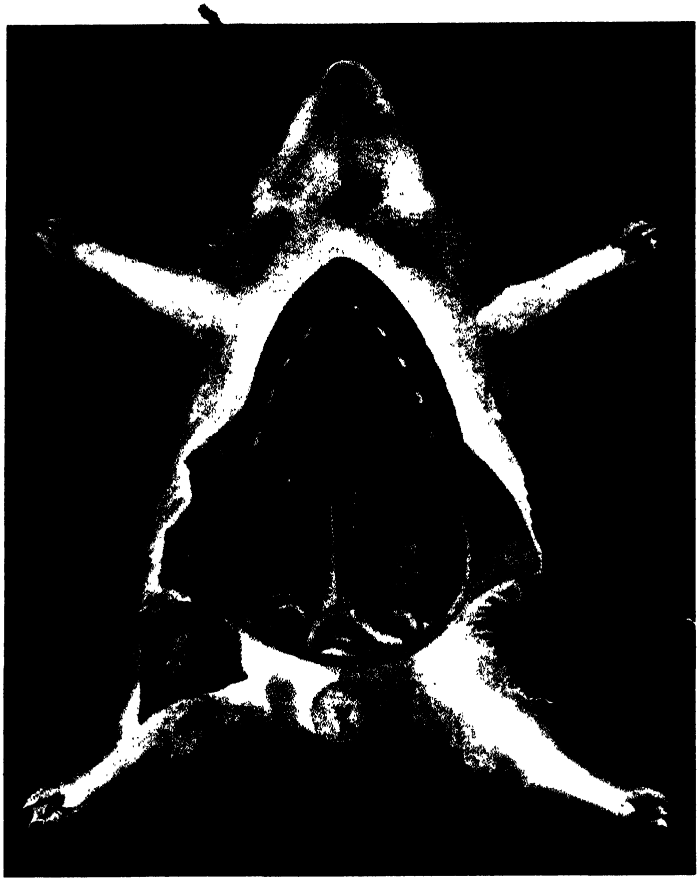


Fig. 197.

Post mortem appearance of a guinea-pig. Inoculated subcutaneously with a virulent culture of *Corynebacterium diphtheriæ*. A thin greyish white membrane along with intense hyperæmia has developed at the site of inoculation. The adrenals are found to be enlarged, swollen and hyperæmic, one of them has been cut open to show the occurrence of hæmorrhages in its substance. A smear examination of the exudate from the local lesion revealed the presence of C. diphtheriæ. Culture from heart's blood was negative. showing the absence of bacteriæmia.

injection of 500 units of diphtheritic antitoxin on the day before the test. This animal serves as a control. Both the animals, after shaving or depilation, are injected intradermally with 0.2 c.c. of the standardised emulsion. Six hours after the injection, the test animal, i.e., the unprotected one, is injected intraperitoneally with 125 units of antitoxin so as to prevent the death of the animal from diphtheritic toxæmia. This, however, does not interfere with the development of reactions. The readings are taken 24 hours, 48 hours and 72 hours after inoculation. If the organism isolated is a virulent C. diphtheriæ, it will produce in the *test animal* a well defined erythematous patch, about 15 m.m. in diameter, which progresses to superficial necrosis. The lesion heals on the third or fourth day. The control animal will not show any sign of illness.

PATHOGENICITY

ANIMALS do not suffer from diphtheria.

In *HUMAN BEINGS*, the organism produces the disease, diphtheria, which is characterised by the formation of a false membrane at the site of the infection and associated with various toxæmic symptoms. Children of 2 to 5 years of age are the commonest victims.

Mode of infection:—In the great majority of cases, the disease is spread from a *carrier*, whether sick or healthy, by droplet infection, the organisms being expelled from the mouth or nostrils by coughing, sneezing, kissing, spitting and even talking.

Fomites, such as objects contaminated by patients, viz., toys, common drinking cups, clothings and beddings, where the bacilli may remain alive for a long period, specially if kept in the dark, are fruitful sources of infection. In schools, the common use of pens, pencils and slates by the scholars, some of whom are carriers, may be responsible for an outbreak. Contaminated *milk* is sometimes responsible for the outbreak of an epidemic, the organism being generally derived from a human carrier.

Animals do not play any part in the spread of the disease, apart from the cow in rare cases. Occasionally, an ulcerative condition of the udder of a cow may be secondarily infected with C. diphtheriæ and the milk of such a cow is heavily infected with the bacilli. Ingestion of such a milk, when imperfectly sterilised, may give rise to an epidemic of the disease. *Cats* were supposed at one time to be responsible for the spread of the disease but the work of *Savage* and others (1919-20) has shown that they are not carriers of C. diphtheriæ.

Portals of entry:—The organisms gain entrance through the nose and nasopharynx which are the favourite sites of their lodgment.

Pathogenesis:—The bacilli thrive in the pharyngeal and laryngeal mucous membranes. They start a superficial inflammation of the epithelium and the tissues underneath, resulting in vascular engorgement and coagulative necrosis of the cells. An exudate, consisting of coagulable lymph and leucocytes, is poured out on this area. It rapidly coagulates and this, together with the degenerated [illegible] leucocytes and the bacilli, constitutes the characteristic pseudo or false membrane” [illegible] 198 and 199). The bacilli

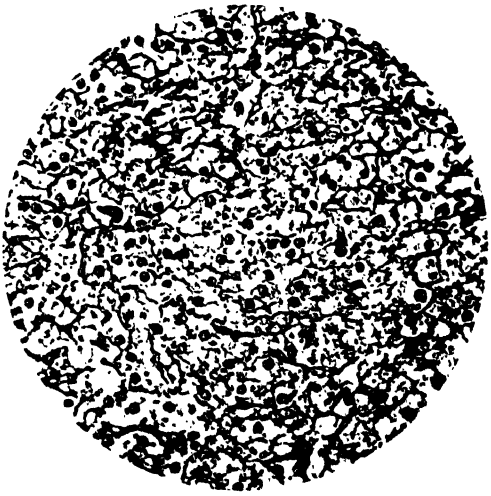


Fig. 198.

Photomicrograph from a section of a *diphtheritic membrane*, showing the characteristic structure. Note the presence of the fibrinous network and inflammatory cells.

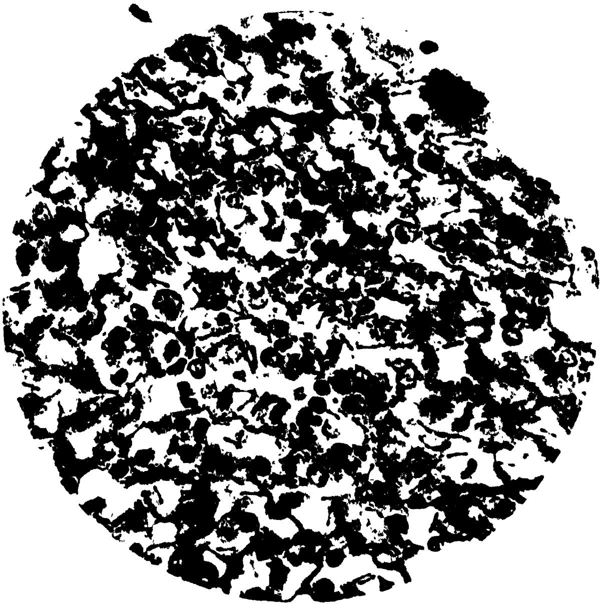


Fig. 199.

A portion of Fig. 198 as seen under the oil immersion lens, showing the presence of a large number of *Corynebacterium diphtheriæ*

multiply in this exudate with incredible rapidity, giving out a large amount of exotoxin which is quickly absorbed into the circulation and produces the characteristic constitutional symptoms. Such pseudo membranes may form anywhere, such as over the tonsils, pharynx, larynx, etc., and usually tend to extend rapidly to the adjacent areas.

Frequent common sites (Fig. 200) are the (i) throat (tonsils and the neighbouring areas), producing faucial diphtheria (this is the commonest site), (ii) nose (inferior turbinate bones), producing nasal diphtheria and (iii) larynx, producing laryngeal diphtheria.

Regional lymph nodes:—Corresponding to the seat of the membrane formation, these are often enlarged and inflamed (adenitis). This is generally due to the absorption of the toxin from the primary

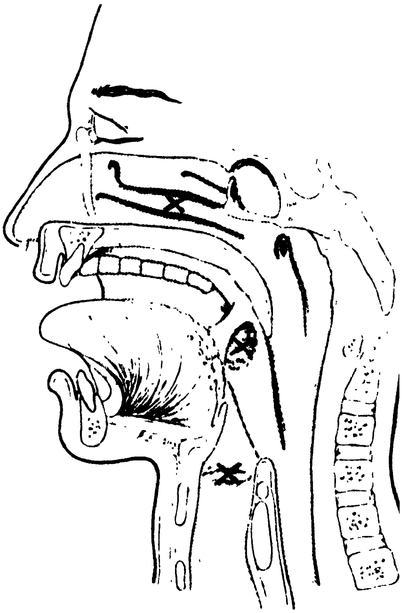


Fig. 200.

Diagram of the upper respiratory tract, showing the common sites, as marked by the sign X, of infection with *Corynebacterium diphtheriæ*.

lesion, but may also be due, to some extent, to an adenitis caused by secondary invaders, the chief of which are the hæmolytic streptococci.

Pathological lesions:—These may be discussed under the following heads:—

A. Effects of the bacilli themselves.

B. Effects of the exotoxin absorbed from the area of the local lesions.

C. Effects of the hæmolytic streptococci as secondary invaders.

A. **Effects of the bacilli themselves.**—A false membrane usually forms at the site of lodgment of the bacilli and as already mentioned, the upper respiratory tract is the commonest and the most frequent site.

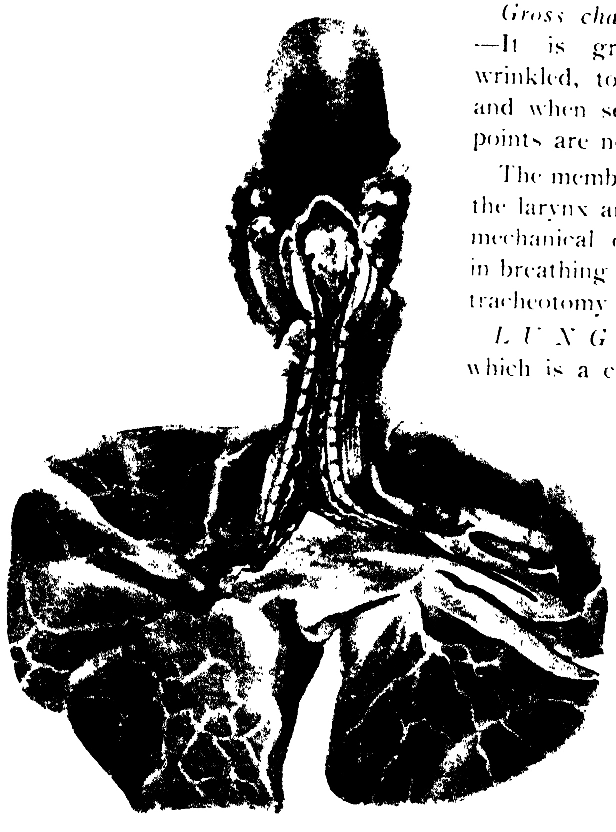


Fig. 201.

Gross specimen of a case of *laryngeal diphtheria*, showing the extension of the false membrane down the trachea and bronchi.

Gross characters of the membrane—It is greyish white in colour, wrinkled, tough, difficult to strip off and when separated, minute bleeding points are noticed.

The membrane which is formed over the larynx and trachea gives rise to a mechanical obstruction and difficulty in breathing (dyspnœa) which requires tracheotomy or intubation.

L U N G S :—Broncho-pneumonia which is a common complication of diphtheria is not produced by C. diphtheriæ but by Streptococci and D. pneumoniæ which are found in the throat as secondary invaders. In cases of laryngeal diphtheria, the membrane can be seen extending downwards (Fig. 201) along the trachea, bronchi and even the bronchioles, producing capillary bronchitis, broncho-pneumonia and collapse of the lungs.

In cases of laryngeal obstruction, emphysematous changes are also seen in the lungs.

B. **Effects of the exotoxin absorbed from the local lesions:—**

The bacilli remain strictly local and ne[illegible]n entrance into the general circulation, i.e., there is no bacteriæmia. [illegible]temic effects and the tissue changes in the internal organs are entirely due to the absorbed exotoxin. These effects are (i) *pyrogenic*, producing fever and general constitutional symptoms; (ii) *tissue poison*, affecting the vital organs, viz., heart, kidneys, adrenals, voluntary muscles, brain, etc.; and (iii) *neurotoxic*, causing degenerative changes in the nerve fibres.

Effects of the exotoxin

HEART:—There is a true myocarditis with hyaline and fatty degeneration of muscle fibres, giving rise to an acute circulatory failure which is generally seen during the *second week and after.* The heart changes are very serious and take a long time to recover. Hence, the value of prolonged and careful convalescence should always be kept in mind in treating cases of diphtheria.

BLOOD VESSELS:—The exotoxin has a marked paralysing effect on the vasomotor control of the blood vessels, producing the condition known as *peripheral failure.*

Note:—In diphtheritic infection, there is often a reduction of blood sugar (hypoglycæmia) which aggravates toxæmia. Another point to be remembered is that a severe grade of anæmia may be produced in this disease.

KIDNEYS:—Albuminuria is very frequent and appears about the *eighth to the tenth day,* but is not usually serious. It is due to a condition of *toxic nephrosis. Acute interstitial nephritis* is not of so frequent an occurrence in India as has been described. Its incidence is very small, occurring only in the gravest type of cases.

ADRENAL GLANDS:—Fatty changes and hæmorrhages are seen and hence, the failure of function. This supra-renal deficiency produces a great fall in the blood pressure.

NERVOUS SYSTEM:—The diphtheria exotoxin produces marked neurotoxic effect on the nerve fibres resulting in paralysis. This generally takes some time and appears *after the third week.*

Incidence—On an average, about 20 per cent. of diphtheria cases show signs of paralysis and it is very common if the nose and throat are both involved; pure nasal and pure laryngeal cases are less frequently followed by this complication. In very severe cases, when the nose and throat are both involved, the incidence may rise as high as 40 per cent. The incidence of this complication also depends on the promptness with which the serum treatment is started. For instance:—

(i) If the serum is given on the first day of the disease, the incidence is 3·6 per cent.

(ii) If the serum is given on the fifth day of the disease, the incidence is 26 per cent.

Pathology:—The lesion is of the nature of a toxic neuritis with parenchymatous degeneration of the peripheral nerves. Inflammatory exudates in between the nerve fibrils compress the latter and cause temporary neuritic symptoms which may clear up as soon as the exudates are absorbed. If the exudate is large and becomes organised, then the symptoms may remain permanent.

Nerves affected:—The *third* and *sixth* cranial nerves are commonly involved. This produces paralysis of the intrinsic and extrinsic muscles of the eyeball. Hence cycloplegia and squint are often noticed. Affection of the *ninth* (glosso-pharyngeal) and *tenth* (vagus) cranial nerves is also very common, resulting in nasal regurgitation of food, nasal intonation and even loss of voice, difficulty in swallowing and rarely dyspnœa. Occasionally, the *phrenic nerve* may be affected, producing paralysis of the diaphragm (see-saw respiration). *Peripheral nerves* of any part of the body may be involved.

C. Effects of hæmolytic streptococci:—In diphtheria, as in any other ulcerative condition of the throat, the hæmolytic streptococci are often found as secondary invaders. The effect of such a secondary infection is shown by a high temperature, broncho-pneumonia and swelling of the upper cervical lymph nodes. In the last situation, the organisms give rise to an acute pyogenic inflammation, ending in suppuration. Such a purulent lymphadenitis is frequently found to persist for a long time after the primary condition in the throat has disappeared under specific treatment. Unless the pus is evacuated, the convalescence becomes prolonged and tedious. The organisms are recovered in pure culture from the pus.

It will thus be seen that the hæmolytic streptococci may, sometimes, complicate a case of diphtheria and add to the seriousness of the disease. For instance, in cases of diphtheria where the temperature runs high and symptoms of general toxæmia are very intense, inspite of the specific serum (anti-diphtheritic) treatment, the possibility of a superimposed streptococcal septicæmia should always be kept in mind. The writers have observed that the use of anti-streptococcal serum (hæmolytic strain) in such cases has been attended with very favourable results.

Local forms of diphtheria:—These local forms are of infrequent occurrence and the following have been described:—

Fibrinous rhinitis (nasal infection)—This condition is characterised by its mildness and by being free from any toxæmic symptoms of diphtheria. The fact that constitutional symptoms may be slight or absent, suggests the presence of some factor which prevents free absorption of the toxin from the nasal cavity (Andrews 1923).

Diphtheritic conjunctivitis:—This is the result of direct inoculation, but may extend from the nose through the naso-lachrymal duct (*vide* Fig. 200).

Diphtheria of the genital tracts (diphtheritic vulvo-vaginitis)—The infection is generally secondary to faucial diphtheria [illegible] organisms being conveyed by contaminated fingers. On rare occas[illegible] may be primary. A false membrane may form on the inner surface o[illegible] or both labia and may extend into the vagina. The condition may be [illegible] with gonorrhœal or other infections. The diagnosis is settled by [illegible]oscopic examination of a smear made from the lesion.

Diphtheritic infection of wounds—This is not very common and for its diagnosis a bacteriological examination is essential.

Otitis media—This may arise as a complication of faucial diphtheria, the infection being spread through the Eustachian tube (*vide* Fig. 200).

Larval diphtheria:—This term is applied to cases of diphtheria unaccompanied by any toxic signs. Virulent bacilli may, however, be present in the lesion and the cases being ambulatory, form a considerable danger to the community.

CARRIERS

These are persons who harbour virulent C. diphtheriæ in their throat or nose, without any manifest sign of the disease. There are two types of carriers, (a) contact and (b) convalescent. The former are those who do not suffer from the disease but have become infected from contact with a case or with another carrier. The latter are those in whose nose and throat the bacilli persist after recovery from the disease.

Thomson, Mann and Marriner observed the following, regarding the disappearance of the bacilli from the throat and nose after the commencement of convalescence. Amongst the throat cases, 50 per cent. become free within a *fortnight,* the remaining 30 per cent. become free within a *month* and the rest become free by the *third month*. Nasal cases tend to clear out somewhat more slowly.

As a rule, the bacilli disappear from the throats within a **fortnight to three weeks** after the separation of the false membrane, but in a small proportion of cases, they may be found for several weeks or months afterwards. Such individuals, harbouring the bacilli, become *convalescent carriers*. Carrier condition tends to persist in younger patients and particularly when the nose is affected along with the throat. Persistence is aided by abnormal conditions of the nose and throat, such as a chronic rhinitis or tonsillitis.

Detection of carriers:—This comprises the following:—

1. Isolation of the C. diphtheriæ from the throat or nose and testing their virulence on guinea-pigs.

2. Schick test—The carriers give a negative reaction. This indicates that the organism in the throat of the carrier is a virulent one (*vide infra*).

Treatment of carriers:—Application of ordinary antiseptics in the throat is useless, as the bacilli lodge in the crypts of the tonsils. Various forms of general specific treatment, e.g., the administration of toxin-antitoxin mixture, toxoid and toxoid-antitoxin floccules, have been tried, but their value is very difficult to assess, as the carriers naturally tend to free themselves of bacilli without any special treatment. Local application of cultures of Staphylococcus pyogenes aureus, to produce an inflammatory reaction, has also been tried. Spraying the throat with pyocyanase had been tried, but the results were not encouraging. Surgical treatment, to correct the abnormalities of the throat and nose, may be resorted to. An unhealthy, chronically inflamed tonsil should be enucleated. In the case of a nasal carrier, irrigation of the nose with alkaline douches yields excellent results.

THERAPEUTIC CONSIDERATIONS

Serum Therapy: —Antitoxin is very largely used now-a-days for the treatment of diphtheria and its introduction in therapy has greatly lowered the mortality and also modified the course of the disease. The impossibility of preventing the disease spreading to the larynx, the extreme rarity with which laryngeal cases recovered without operation and the high death rate from tracheotomy, as well as the inability to cut short a faucial attack, made the prognosis in the pre-antitoxin days much more grave than at present. With the introduction of the antitoxin, however, not only the incidence and mortality of laryngeal cases have fallen considerably, but the proportion of such cases in which operation is necessary has been greatly reduced and the recovery rate after tracheotomy or intubation enormously increased. The following table is very instructive, comparing the case mortality and recovery rate during pre-antitoxin days and present days (antitoxin):—

	Pre-antitoxin day (before 1894).	Present day.
...	30 to 40 per cent.	8 to 10 per cent.
Mortality rate in laryngeal cases. ...	60 to 70 per cent	10 to 15 per cent.
Recovery rate from Tracheotomy. ...	25 to 30 per cent.	70 to 75 per cent.

Dosage:—The amount of antitoxin to be given in a particular case depends on (i) the severity of the disease and (ii) the day of the disease when treatment began, and not on the age of the patient. Thus, if seen on the first day of the disease, 6,000 to 12,000 units are enough for *mild cases,* whereas 12,000 to 20,000 units should be given for *cases of medium severity.* For *bad cases,* 24,000 to 30,000 units should be considered an effective dose. After the first day of onset, however, these doses should be increased.

Regarding the question whether a single dose or repeated doses should be administered, the general aim is to give all the necessary antitoxin within twenty-four hours. It should be noted that combination of toxin with the tissue is gradual and is not completed at once. In the early stages, the toxin remains in a state of loose and dissociable combina[illegible] but later, a final and stable union takes place between the toxins and ti[illegible] producing the pathological effects. Hence, early treatment with a ma[illegible] dose is of paramount importance. This will tend to dissociate the loo[illegible]-tissue combination and the free toxin will be at once neutralised by th[illegible] amount of antitoxin introduced, thus preventing its pathological effects on vital organs. Repetition of the dose is guided by the general condition of the patient and the rapidity with which the membrane separates.

The value of early treatment is shown by the following statistics from the Annual report of the Metropolitan Asylums Boards, London, for 1908.

Day of disease on which treatment began.	*Percentage mortality.*
First day	3·0
Second day	6·5
Third day	10·6
Fourth day	12·9
Fifth day and afterwards	14·8

Methods of administration:—The antitoxin may be injected subcutaneously, intramuscularly, or intravenously. The object of the antitoxin treatment is to have the maximum concentration of the remedy in the blood without the least possible delay. The time required for the appearance of the maximum concentration in the blood by the various methods is as follows, intravenous, at once; intramuscular, 24 hours and subcutaneous, about 3 days. For therapeutic purposes, therefore, the subcutaneous method should not be resorted to and the commonly accepted method is the *intramuscular inoculation,* generally given in the outer part of the thigh. Intravenous route is left for very bad and late cases.

The strength of the antitoxin is expressed in "units" and one unit is defined as the amount necessary to neutralise 100 M.L.D. of diphtheritic toxin for a standard guinea-pig. Preparations of concentrated antitoxin are available in the market and can be utilised where large doses are required. The advantage of using such a concentrated antitoxic serum is that very little foreign protein is injected into the patient and consequently the chances of anaphylaxis and other unpleasant serum reactions are minimised.

Mode of action of antitoxins:—As mentioned on previous occasions, introduction of antitoxin therapy cannot repair the damage that has already been produced, but can prevent further injury being inflicted on tissues. Hence, the early treatment is so seriously stressed upon. Signs and symptoms of diphtheria are the manifestations of toxæmia and, as there is no bacteriæmia, the question of bactericidal action by the serum does not come in. The toxins which are circulating in the blood are neutralised by the antitoxin and those that are in loose combination are dissociated and rendered inert. The toxins which are fixed in the tissues are not influenced in any way. Thus, the important effect of serum therapy is to relieve the toxæmia, thereby allowing the natural defensive powers of the body to fight with the organism at the site of the membrane formation. The disappearance of the membrane resulting from antitoxin treatment is not due to any specific lytic actions of the antitoxin, but is effected by the usual reparatory processes of the body.

Prophylactic immunisation against diphtheria

Methods:—

1. *Passively—By injection of antitoxin.*
2. *Actively—(a) By toxin-antitoxin mixture (Park), (b) by toxoid or anatoxin (Ramon) and (c) by toxoid-antitoxin floccules.*

Passive immunisation:—The use of diphtheria antitoxin, as a prophylactic measure, is not very advantageous. The immunity produced is of a very brief duration and does not last for more than 3 weeks. This method is often utilised to produce a rapid, though temporary, immunity. The usual dose for this purpose consists of 500 units for children and 1,000 units for adults. It is the opinion of many authorities that susceptible contacts should not have any prophylactic antitoxin immunisation but they should be isolated and carefully observed for the development of clinical signs of diphtheria. As soon as a positive clinical diagnosis is made, antitoxin should be administered at the earliest moment.

Objections for antitoxin prophylaxis:—

1. Short duration of protection.
2. Renders subsequent therapeutic injection less effective, owing to the development of precipitins in the blood.
3. Risk of anaphylaxis during subsequent serum treatment.
4. Carriers of virulent bacilli are created by this passive immunisation.

Active immunisation (Diphtheria prophylactic):—Susceptible individuals, as proved by positive Schick reaction (*vide* p. 357), may be actively immunised by the injection of any one of the following preparations—Park's toxin-antitoxin mixtures (T.A.M.), Ramon's anatoxin or formolised toxin (F.T.) and toxoid-antitoxin floccules (T.A.F.).

(i) *By toxin-antitoxin mixture*—The preparation is standardised to contain 3 L+ doses of toxin (vide p. 158) and 3 units of antitoxin. Such a mixture will retain its properties unimpaired for some months, but it should preferably be used within 3 months of its issue. When kept in the cold, the antitoxin is dissociated, rendering the mixture much more toxic than it should be; hence, frozen mixtures are dangerous and should always be avoided. Owing to this disadvantage, it has been replaced by toxoid-antitoxin mixtures and other diphtheria prophylactics.

Three subcutaneous injections of 1 c.c. each, at intervals of 7 to 10 days, are required for immunisation. Immunity developed is a gradual one and takes at least 3 months and is complete in 3 to 6 months, as shown by a negative Schick test. The duration of the immunity induced by this method is also a protracted one and lasts for more than 6 years in 90 per cent. of children. The antitoxin formed in the individual, being of his own manufacture, persists much longer than when foreign antitoxin is injected.

(ii) *By Ramon's anatoxin or formolised toxin* (F.T.)—Anatoxin is a form of toxoid, obtained by the action of formalin (0·4 per cent.) on diphtheria toxin and incubating at 37°C. for one month. It has recently been used as a substitute for toxin-antitoxin mixture and has the great advantage of being non-toxic. Such an anatoxin has lost its toxophore group, but still retains its haptophore one which is thus capable of provoking an antibody response. Three subcutaneous injections of 0·5, 1 and 1·5 c.c. are made at intervals of 2 to 3 weeks.

(iii) *By toxoid-antitoxin floccules* (T.A.F.)—This consists of a suspension of flocculent precipitate formed by neutralising equal proportions of toxoid and antitoxin. This is a costly preparation, but its immunising efficiency is very high.

Note:—It has been remarked by Glenny and his co-workers that owing to the rapid elimination of toxoid and the floccule, they do not act as efficient and continuous stimuli. But when they are treated with alum, their antigenic efficiency is much increased.

LABORATORY DIAGNOSIS

Diphtheria is a disease, the diagnosis of which mainly rests on the physician and not on the bacteriologist. This is because complete laboratory diagnosis is not possible within 24 hours and owing to the seriousness of the condition, the antitoxin treatment cannot be held back. A microscopic and cultural report can, however, be given within 12 hours, but the mere presence of the bacilli does not prove that the case is one of diphtheria. Bacteriologist can only report their presence or absence and it is the physician who will associate the presence of the organism with the disease and assess its importance. Whenever there is any suspicion of a case being diphtheria, it is the duty of the physician to start the antitoxin treatment at once, without waiting for the laboratory report. It is in those cases where the suspicion is against diphtheria and the material is obtained from the throat of a supposed carrier or from other places of the body, from animals, and in fact from any source apart from a human being, that a full laboratory diagnosis is necessary.

Lastly, to give proper facilities to the bacteriologist, the latter should be provided with a detailed information about the history of the case, and the nature and source of the material submitted.

Collection of the material and transmission:—It is apparent that the best material will be a piece of the membrane obtained with a pair of forceps. Sterile swabs, introduced by v. Esmarch in 1895, will also serve the purpose of obtaining the membrane.

Essentials of a throat swab (Fig. 202)—

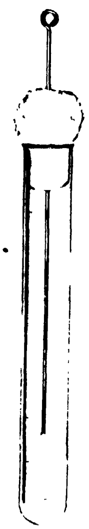


Fig. 202. Throat swab.

(i) Non-absorbent cotton wool.

(ii) Copper wire—The advantage of it is that it can be bent in any direction required for the purpose.

Important hints for taking swabs:—

(a) Sufficient assistance.

(b) Spatula or spoon.

(c) Good view of the throat—use of sunlight or artificial light (torch or hand lamp).

(d) Always try to take out some membrane with the swab.

(e) No antiseptics in the form of gargles or sprays should be applied before the swab is taken.

In cases of faucial diphtheria, the membrane can be seen and a swab is taken from it.

In cases of laryngeal diphtheria, however, the best thing will be to take a laryngeal swab, but the bacilli are also present in the fauces; hence, a thorough rubbing of the swab over it will serve the purpose. In nasal diphtheria, the swab should be so introduced as to reach the turbinate bones, because these are the usual seats of membrane formation.

Note:—Immediate inoculation offers the best chances of success. But as the C. diphtheriæ resists considerable drying, successful cultures are obtained even after hours.

Methods of diagnosis:—

These include the following:—

(1) **Direct film examination (microscopical)**:—The organism can be identified from its morphological characters and a positive result is obtained if a large number of bacilli are present or membrane available. A presumptive diagnosis can be given from such an examination, particularly if the swabs are taken from children suffering from acute sore throat. *Negative result has no value.*

(2) **Cultivation**:—The media used for the purpose are serum agar, Loeffler's serum medium, Dorset's egg medium and tellurite medium. In a suspected case, where a microscopical examination has failed to demonstrate the presence of the organism, cultures should always be made. After 6 to 12 hours' incubation, the growth can be recognised by its characteristic appearance *(vide supra)*, but it is always advisable to examine a stained smear.

Note:—In most cases, the diagnosis can be [illegible] n from microscopical and cultural examinations alone, but for a comp [illegible] agnosis (specially in cases of doubt), the other methods, such as ferm [illegible] n reaction and virulence test, are performed.

(3) **Biochemical reaction**:—The various fermentation reactions that are carried out are shown in the table below and for the sake of comparison, the fermentation reactions of other group of organisms are included in the table.

Organisms.	Glucose.	Galactose.	Saccharose.	Dextrin.
C. diphtheriæ ...	+	+	o	+
C. hofmanni ...	o	o	o	o
C. xerosis ...	+	o	+	o

+ = Acid only; o = No change.

(4) **Guinea-pig inoculation**:—This should always be resorted to in the diagnosis of carriers and also when C. diphtheriæ is isolated from lesions other than acute sore throat. The organism is isolated in pure culture by subculturing on to serum agar plates and then injected into guinea-pigs for testing its virulence (*vide supra*).

Note:—Although there is a moderate leucocytosis and hypoglycæmia, blood examination is of no value in the bacteriological diagnosis of diphtheria. This is because neither there is any important serological reaction nor can the organism be isolated by blood culture.

SCHICK REACTION

In the year 1913, Bela Schick of Vienna devised this test to determine the susceptibility of individuals to diphtheria.

Principle:—It has been observed that the presence of 1/50 to 1/20 unit of diphtheritic antitoxin per cubic centimetre of blood gives immunity to diphtheria. Such an amount of antitoxin, if present, prevents any reaction after injection of 1/50 M. L. D. of diphtheria toxin. This amount is often present in normal persons who have acquired it from a preceding infection with C. diphtheriæ, with or without any accompaniment of the disease. The newborn infant obtains it from its mother's blood through the placental circulation and the immunity thus acquired, gradually and progressively diminishes after a year, unless it is again supplemented or increased by subminimal infection. Hence, the greatest incidence of the disease is after one year when the inherited immunity almost wears off.

Materials required:—

1. **The test toxin**—This is the standard diphtheritic toxin of which 0·2 c.c. is equivalent to 1/50 M.L.D.
2. **Control toxin**—This is prepared by heating the standard toxin to 70°C. for 5 minutes. This process destroys the toxophore group of the toxin molecule.

Technique:—After cleansing the flexor surface of the forearm with alcohol, 0·2 c.c. (1/50 M.L.D.) of the standard diphtheritic toxin is injected *intradermally* (not hypodermically), so as to raise a definite bulla. A 1 c.c. syringe with a sharp fine needle may be employed for the purpose and after washing it or preferably using another syringe, an equal amount of the control toxin (heated toxin) is injected in a similar manner into the opposite forearm.

Results:—Four varieties of reactions may be observed—negative, positive, pseudo and combined.

(i) **Negative reaction**:—This is indicated by the absence of any reaction on either arm.

Remarks:—Such a result shows that the toxin is neutralised by antitoxin which is present in sufficient amounts in the blood of the individual to render him immune to diphtheria.

(ii) **Positive reaction**:—This is indicated by the appearance of a pink halo around the site of the injection and the development of an œdema at the end of 24 to 36 hours. On the fourth day, the swelling becomes maximum. On the seventh day, the reaction gradually fades showing superficial scaling, leaving a small amount of brownish pigmentation for weeks. The control injection on the other arm shows no redness or swelling.

Remarks:—These persons have not sufficient amount of antitoxin to neutralise the injected toxin, and hence, they are susceptible to diphtheria infection.

(iii) **Pseudo-reaction**:—This is evidenced by an area of redness which is often urticarial and indurated. It appears early (within 6 to 12 hours) and disappears mostly by the fourth day, leaving no pigmentation. It occurs equally in both arms.

Remarks:—Such a reaction is due to the presence of bacterial proteins in the toxin preparation. The interpretation is that the individual is immune to diphtheria.

(iv) **Combined reaction (pseudo and positive)**:—Reaction occurs in both arms. A pseudo-reaction in the control arm, whereas on the test arm a combination of pseudo and positive reactions.

Remarks:—The reaction indicates that the individual is susceptible to diphtheria.

Note:—The important readings are the *fourth day* and the *seventh day* ones. The pseudo reactions will disappear mostly by the fourth day, while the positive reaction will persist till the seventh day.

Value of the test:—

1. A positive Schick test signifies that the individual is susceptible and requires immunisation.

2. A negative Schick test shows individua ..nity. It should be noted carefully that the carriers of virulent C. ..riæ always give a

negative Schick reaction. Thus, it may be used to differentiate carriers of C. diphtheriæ from cases of diphtheria, for the former react negatively, while the latter react positively.

3. This also provides a means of determining the virulence of an organism isolated from a suspected carrier. If the supposed person gives a positive Schick reaction, it signifies that the organism is avirulent.

DIPHTHEROID OR DIPHTHERIA-LIKE ORGANISMS

The term "*Diphtheroid bacilli*" is applied to a large heterogeneous group of organisms held together by morphological and superficial cultural similarity and largely consists of saprophytes. They are so called because of their morphological resemblance to C. diphtheriæ. All the members of this family are Gram-positive, non-motile and non-sporing bacilli. These are probably harmless parasites in the human and animal body. The organisms are widely distributed and have been found in the following places:—Nose and throat, eyes, urogenital tract, skin, surgical wounds, certain pathological exudates and lymph nodes in Hodgkin's disease. Thus, they are so ubiquitous in their distribution that their association with any specific disease must be conservatively assessed. The two most important properties by which these diphtheroid organisms are differentiated from the true C. diphtheriæ are:—(i) sugar fermentation (*vide* p. 356) and (ii) animal pathogenicity (guinea-pig).

Corynebacterium hofmanni (*B. hofmanni*)

Isolated by von Hofmann in 1888, from throats of normal persons.

Habitat—It is a common commensal of the throat, nose and nasopharynx.

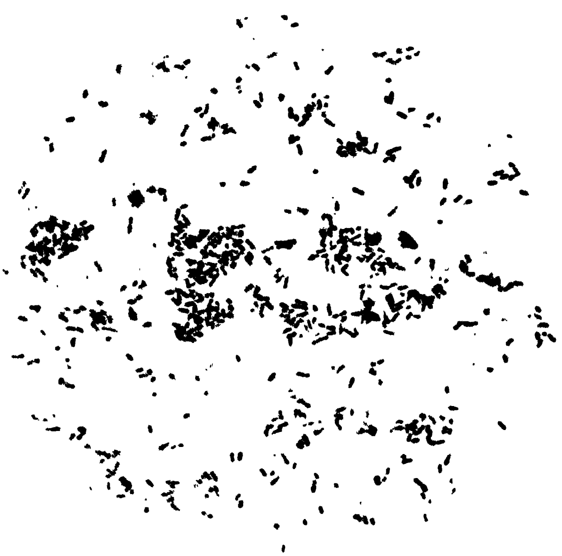


Fig. 203.
Corynebacterium hofmanni.

Morpholgy—It is shorter and thicker than C. diphtheriæ. It possesses an unstained single transverse band which gives the bacillus a diplococcoid appearance (Fig. 203).

Involution forms are as a rule not met with.

Staining reaction—Metachromatic granules, though not a characteristic feature, are sometimes encountered.

Culture—It is a strict aerobe. It grows readily on ordinary agar.

Biochemical reactions—It does not ferment any sugar, such as glucose, saccharose, dextrin and others.

Toxin production—It does not form any exotoxin nor any hæmolysin.

Pathogenicity—Non-pathogenic to guinea-pigs.

Corynebacterium xerosis (*B. xerosis*)

Isolated from the conjunctiva by Reymond in 1881 and described fully by Kuschbert and Neisser in 1883.

Habitat—A commensal of the conjunctival sac.

In *morphology* and *staining reactions*—it closely resembles C. diphtheriæ (Klebs-Loeffler bacillus). It does not usually show the characteristic metachromatic granules.

Culture—The growth is very similar to that of C. diphtheriæ. In primary cultures, colonies take some time to appear (over 30 hours).

Biochemical reactions—It produces acid in media containing glucose, maltose and saccharose.

Pathogenicity—It is not pathogenic to guinea-pigs.

Corynebacterium acnes (*Acne bacillus*)

Unna, in 1896, described this organism in the lesions of cutaneous acne. It was isolated in culture by Sabouraud in 1902.

Morphology—It is a short thick bacillus, frequently clubshaped.

Culture—It grows only under anaerobic or partial anaerobic conditions. An acid medium is to be preferred, such as a reaction of pH 6·8 to 6·2.

It does not grow in ordinary agar. It has been found that broth to which is added 1 per cent. glucose and 1 per cent. oleic acid, the whole being covered with a layer of oil, is a satisfactory medium.

Fleming (1909) employed ordinary nutrient agar with an addition of 1 to 5 per cent. of oleic acid.

Biochemical reaction—It ferments glucose, but may or may not ferment saccharose.

Pathogenicity—Pathogenic to mice by intraperitoneal inoculation. In man, it is supposed to be the cause of comedo, [illegible] suppuration is due to a secondary infection by the staphylococcus. [illegible] is a small, black-tipped, worm-like mass which is found on the fa[illegible]me persons.

TABLE SHOWING THE DIFFERENTIAL CHARACTERS OF THE ORGANISMS OF THE CORYNEBACTERIUM GROUP.

Organisms.	Hiss's serum water medium plus 1 per cent.					Metachromatic granules	Oxygen requirement
	Glucose.	Maltose.	Saccharose	Dextrin	Mannite		
C. diphtheriæ ...	+	+	–	+	–	+	Aerobic
C. hofmanni ...	–	+	–	–	–	–	Aerobic
C. xerosis							Aerobic
C. acnes			+	Doubtful			Microaerophilic.

Organisms.	Production of hæmolysin	Toxin production	Pathogenicity	Normal habitat and source
C. diphtheriæ ...	+ (Mitis type)			Parasitic in human beings; found in nose and nasopharynx of carriers.
C. hofmanni ...				Common commensal of throat, nose and nasopharynx.
C. xerosis				Commensal of the conjunctival sac.
C. acnes			Doubtful	In cutaneous acne, either alone or in association with Staphylococcus.

+ = Positive ; – = Negative.

CHAPTER XXV

SPORE-BEARING BACTERIA

The bacilli which have the power to produce spores (endospores), when placed under adverse circumstances, are referred to as spore-bearing bacteria. For further details about spore formation, *vide pages* 5 to 7.

All the spore-bearing bacteria are Gram-positive. The whole group is divided into two main subdivisions.

I. Those which grow aerobically—*BACILLACÆ.*

II. Those which grow anaerobically—*CLOSTRIDIUM.*

BACILLACÆ

(Aerobic spore-bearing bacteria or Spore-bearing aerobes.)

This group includes mostly saprophytes; the type species being Bacillus subtilis. The only pathogenic member of the group is Bacillus anthracis.

Organisms belonging to this group are:—

A. ***Pathogenic forms,*** e.g., *B. anthracis* (non-motile).

B. ***Non-pathogenic forms*** or saprophytes (motile), e.g., *B. subtilis* (Hay bacillus), *B. mesentericus vulgatus* (Potato bacillus), *B. megatherium* (B. anthracoides or B. pseudoanthracis) and B. *mycoides* (Root bacillus). All these organisms are constantly present in the dust, soil, hay and decomposing vegetable materials.

BACILLUS ANTHRACIS

HISTORICAL:—The organism was first discovered in the blood of an infected cow by *Pollender* in 1849. *Davaine* (1850) also observed these organisms and recognised their true nature in the year 1863. The introduction of solid medium and plating methods for the isolation of pure cultures by *Robert Koch* (1875) had made it possible to obtain this organism in pure culture and to reproduce the disease in animals by experimental inoculation. *Pasteur,* in 1881, first demonstrated the prophylactic value of an attenuated organism by protecting sheep against anthrax. Special historical interest is therefore attached to the anthrax bacillus for the following reasons :—

(i) It was the first pathogenic bacillus to be se [illegible] er the microscope *(Pollender 1849).*

(ii) It was the first communicable infection [illegible] experimentally transferred *(Davaine 1850).*

(iii) It was the first bacterium to be grown in pure culture *(Robert Koch 1875)*.

(iv) It was the first organism in connection with the demonstration of the prophylactic value of an attenuated vaccine *(Pasteur 1881)*.

HABITAT:—It is parasitic in man, cattle, sheep and other laboratory animals.

MORPHOLOGY:—

Shape—It is a thick, rod-shaped organism, with square-cut or concave ends (rectangular bacillus) and thus it is in sharp contrast to the rounded ends of many other bacilli.

Size—It is fairly large and is perhaps the largest bacillus of importance in human pathology. It measures about 5 to 8 μ in length by 1 to 1·5 μ in breadth.

Arrangement—It is commonly arranged in chains (Strepto-bacillus). In blood and tissues, the chains are small, consisting of 5 to 10 segments, but in cultures, long chains are found consisting of hundreds of segments. When the bacilli are in chains, they touch each other only at the ends of their corners, leaving an oval chink between the ends of the organisms, the appearance resembling a rod of bamboo (Fig. 204 a).

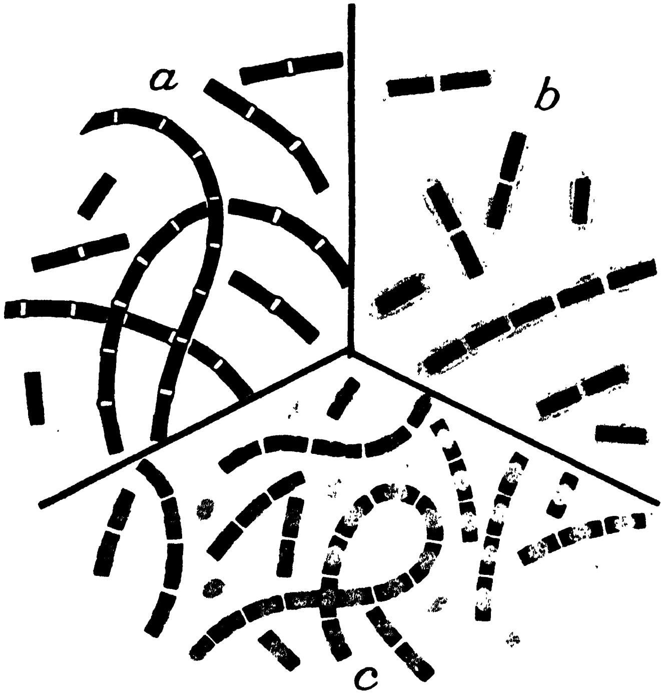


Fig. 204.

Morphology of *Bacillus anthracis*.

a, bacilli in chains; b, capsules surrounding the bacilli; c, bacilli with oval and equatorial spores.

Spores—B. anthracis is a sporing organism. The spores are never met with inside the body of an animal suffering from anthrax, because the bacilli do not sporulate as long as free access of air is prevented. An adequate supply of oxygen and an optimum temperature of 30°C. are necessary for the formation of spores.

The spores appear as refractile spots in the centre of the bacilli when they are examined unstained. They increase in size till they appear as oval structures located in the middle of the bacillary protoplasm (equatorial and oval spores—*vide* Fig. 204 c). They are of the same width as the bacilli and measure about 1·25 μ by 1 μ (compare the Clostridia in which the diameter of the spores is larger than that of the bacillary body). After the spores are fully formed, the residual protoplasm of the bacilli disappears and the spores are left as free structures (mature spore).

Spores represent a highly resistant phase in the life history of the organism, so that they can survive under conditions which are unfavourable to the vegetative phase. Thus, the object of spore-formation is to preserve the race in adverse circumstances. Placed under suitable conditions of warmth, moisture and food supply, spores germinate. The capsule of the spore ruptures at one pole and the protoplasm slips out of the spore capsule (polar germination—*vide* p. 7), taking the rod-shaped form of the vegetative phase.

Capsules—Ordinarily, no capsules are observed. A capsule may, however, be noted in preparations from tissues and blood of an infected animal. The capsule is continuous over the whole chain (*vide* Fig. 204 b).

Flagella and motility—The organism does not possess any flagella and is devoid of any motility.

STAINING REACTIONS:—This organism stains well with ordinary aniline dyes and is strongly Gram-positive. Spores are not stained with ordinary aniline dyes, but may be demonstrated (Fig. 205) by any of the usual spore-staining procedures (*vide page* 29).

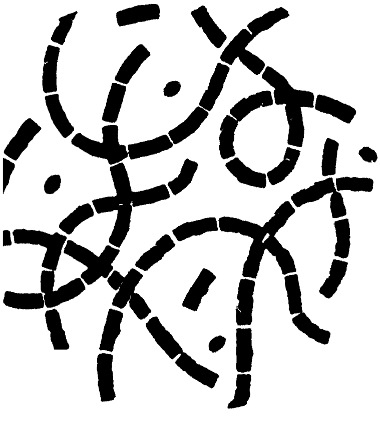


Fig. 205.

Spores of *Bacillus anthracis* stained by modified Ziehl-Neelsen method. Spores are stained red and the bacillary protoplasm blue.

CULTURAL CHARACTERS:—It is an aerobe and facultative anaerobe. The optimum temperature for growth is 37°C. It grows well in all ordinary media.

On agar—The colonies are greyish white and form circular discs with an irregular wavy margin (Fig. 206). When examined microscopically, they have a "*curled hair-lock*" appearance (Fig. 207). These wavy wreaths are made up of long continuous convoluted filaments, lying parallel with one another, each filament representing a chain of bacilli.

In broth growth takes place rapidly, forming a floccular deposit at the bottom, consisting of interwoven threads. There is no uniform turbidity, the upper part of the broth being almost clear (*cf.* B. subtilis in which there is a scum or pellicle on the surface with uniform turbidity).

Gelatin—In stab-culture, a characteristic appearance is seen. The growth appears at first as a thin white line along the track of the needle puncture, from this, growth proceeds in fine lateral spikes, radiating in all directions at right angles to the central stem. These spikelets are longest at the upper part of the needle track, owing to more active growth in well oxygenated environment. The whole picture resembles that of an "*inverted fir-tree*" (Fig. 208). Later, liquefaction occurs, commencing from the top of the growth and gradually spreading down the stab, until the whole of the gelatin is liquefied.

BIOCHEMICAL REACTIONS:—B. anthracis ferments glucose, saccharose and maltose with the production of acid but no gas.

Little or no hæmolytic action is exerted on a blood agar plate.

Antagonism between Pseudomonas æruginosa (B. pyocyaneus) and B. anthracis:—The former has a destructive influence on the growth of the latter, when allowed to grow together. "Pyocyanase," a ferment-like body, which can be isolated from cultures of Ps. æruginosa, is found to possess curative and preventive properties against anthrax infection.

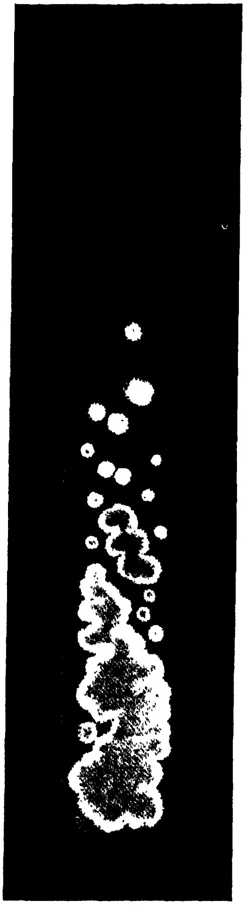


Fig. 206.
Surface colonies of *B. anthracis* on agar.

RESISTANCE: — Vegetative forms of anthrax bacilli are even less resistant than those of other non-sporing bacteria. An exposure for ten minutes to a temperature of 55°C. is sufficient to kill the bacilli. Resistance to disinfectants is also poor, an 1 per cent. solution of carbolic acid quickly destroying the bacilli. Spores are, however, extremely resistant to physical and chemical agencies.

Fig. 207.
Growth of *B. anthracis* on agar. Note the curled hair-lock appearance of the growth.

Drying—The spores, particularly when dried, are very hardy and may remain alive for years under adverse circumstances. Thus, it can remain viable for 12 to 15 years in a dry soil.

Sunlight—Direct sunlight destroys anthrax spores within 6 to 12 hours.

Heat—Moist heat is much more effective than dry heat in killing spores. Boiling in water kills them in 10 minutes and an exposure to live steam at 100°C. kills them in 5 to 10 minutes; whereas dry heat at 140°C. requires an exposure of 3 hours to destroy them.

Antiseptics—Phenol, in 5 per cent. solution, is not a very effective disinfectant for killing spores, as some strains may retain their viability even after an exposure for 6 weeks. *Corrosive sublimate*, 1 in 2,000, kills most strains in 40 minutes. *Formalin* is one of the most potent disinfectants against anthrax spores, having a selective action on them. It is, therefore, largely employed in the treatment of infected animal products, such as wool and hides.

Table showing resistance of B. anthracis—vide page 6.

VIRULENCE:—Virulence of a particular strain may be lowered or increased in various ways. The lowering of the virulence is effected by the following means:—

(i) By heating the bacilli to 55°C. for 10 minutes.

(ii) By prolonged cultivation (12 to 24 days) at a temperature of 42°C. (Pasteur used such an attenuated strain for his prophylactic vaccination of cattle and sheep).

(iii) By growing the organisms in a medium containing weak disinfectants, such as phenol 1 in 800.

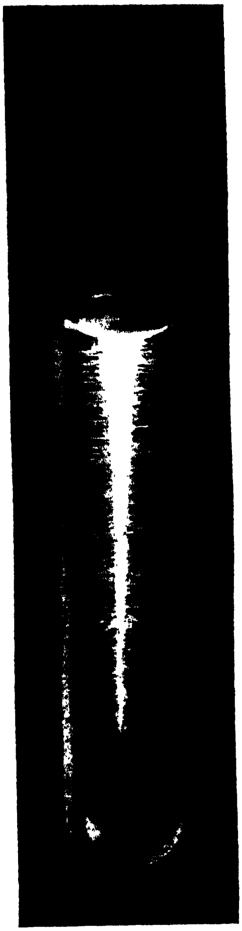


Fig. 208.
Gelatin stab culture of *B. anthracis*, showing the inverted fir-tree appearance of the growth.

Attenuated strains so produced may never regain the power to produce spores and thus nonspore-forming races may be evolved. Once the virulence is reduced, the new grade of virulence remains fairly constant.

The increase of virulence may be brought about by repeated passages through susceptible animals.

TOXIN PRODUCTION:—Neither endotoxins nor exotoxins have ever been satisfactorily demonstrated in cultures of B. anthracis. Neither culture filtrates nor dead bacilli have any noticeable toxic effect when tested on laboratory animals.

SEROLOGICAL REACTIONS:—Precipitins [illegible] und in the immune sera and are useful for diagnostic purposes. An [illegible] of an infected organ, such as the spleen, contains a substance derived [illegible] the bacterial bodies and this interacts with the precipitin in an anthrax immune serum, yielding a precipitate. The immune substance derived from the bacterial bodies is not destroyed by heat, boiling, antiseptics nor even by putrefactive changes. It is for this reason, that it serves as a very good diagnostic test for determining whether a suspected animal died of anthrax infection. This has given rise to **Ascoli's thermoprecipitin reaction.**

The principle of the reaction depends, as stated above, on the formation of a precipitate, when the percipitinogen in the bacterial body is mixed with the precipitin contained in the immunised serum. As the precipitinogen is a very stable substance, not affected by boiling, antiseptics and decomposition, it is conveniently obtained from the spleen of the infected animal by boiling it in saline.

Technique:—A piece of spleen from the suspected animal is boiled with about ten times its volume of normal saline. This is allowed to cool and then filtered. The filtrate is collected in a clean test tube and some immune serum is carefully introduced, by means of a pipette, into the bottom. If a progressively deepening ring of turbidity appears at the junction of the liquids, the reaction is positive and the infection is proved. If no such precipitate forms, the reaction is negative and the animal did not die of anthrax infection. It may be pointed out that this test is useful only after the animal is dead.

ANIMAL INOCULATION:—Of the animals commonly used in the laboratory, mice, guinea-pigs and rabbits are the most susceptible to infection by B. anthracis on subcutaneous inoculation. The tissue of the body most susceptible to anthrax infection is apparently the skin and the conditions for occurrence of infection are specially favourable when the organisms are inoculated into the skin (Besredka's observation).

The susceptibility of mice, guinea-pigs and rabbits varies in the order named and this fact can be utilised to judge the virulence of a given strain of anthrax bacillus. If all the three animals die, it is highly virulent; if mice and guinea-pigs succumb but not rabbits, it is of moderate virulence; if only the mice die, it is of low virulence. If a strain fails to kill all the three animals, it is devoid of any virulence. This method of testing the virulence of a strain is utilised in the preparation of Pasteur's vaccine.

Subcutaneous inoculation of fully virulent bacilli into susceptible animals (mice or guinea-pigs) produces a fatal result within 48 hours.

Post mortem examination reveals the following:—

Local—At the site of the inoculation, an intense inflammatory œdema with hæmorrhagic exudations is seen. Numerous bacilli are found on microscopical examination.

General—The internal organs are congested and hæmorrhagic spots are frequently present on serous membranes. The bacilli are found in large numbers in the internal organs, specially in the spleen, liver and kidneys. The *spleen* is particularly involved and is found to be very considerably enlarged (about 8 to 10 times). It is congested, soft and diffluent, containing enormous numbers of bacilli (Fig. 209). Owing to such marked changes in this organ, the disease is often called " splenic fever." In the *liver,* the bacilli are present in large numbers inside the capillaries and in the *kidneys,* they are markedly found in the glomeruli and the intertubular capillaries.

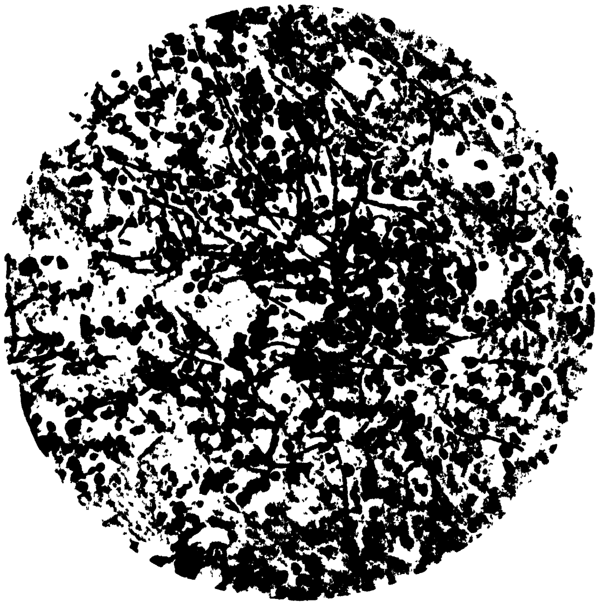


Fig. 209.

Photomicrograph from a section of spleen of a guinea-pig, dead of anthrax septicæmia. Note the presence of a large number of *B. anthracis* in the splenic pulp.

Susceptibility of animals to B. anthracis:—

Very susceptible—Ox, sheep, guinea-pigs and mice.

Less susceptible—Horse, pig, goat and deer.

Almost insusceptible—Birds, fish, reptiles and cold-blooded animals.

Highly immune—Algerian sheep and adult carnivora.

PATHOGENICITY

Anthrax belongs to that group of diseases which occur primarily in animals and only secondarily in man.

Animals:—Anthrax is primarily a disease of the herbivora, occurring epizootically, chiefly in cattle and sheep (except the Algerian sheep), and is of world-wide distribution. The disease is very virulent in these animals in which death occurs with great rapidity. It is usually one of the acute septicæmias and on account of the great enlargement of the spleen (the most marked post-mortem finding), the name "splenic fever" [illegible]iven to the disease.

Mode of infection:—The infection in animal[illegible] place chiefly by the alimentary canal, through the ingestion of s[illegible] along with fodder. The vegetative bacilli are destroyed by the gastr[illegible], whereas the spores remain alive. It has been suggested that dama[illegible]e mucous membrane is essential in anthrax infection and this is effecte[illegible]rticles of indigestible vegetable matter. In the intestines, the spores develop into vegetative forms, multiply and invade the system. Cutaneous infection, similar to that in man, may also take place in animals, but this variety of lesion is much less common than the preceding.

Spread of the disease in nature results from the ingestion of spores during feeding, the transmission among animals being effected by the agency of the excreta or unburnt carcasses of infected animals. The hæmorrhagic discharge that comes out of the mouth, nostrils or the anus of an infected animal contains large numbers of anthrax bacilli which fall upon the ground and undergo sporulation. The spores may remain in the immediate vicinity or may be scattered by rain and winds over considerable areas and thus infect the pasture. The spores may remain alive in this condition for many years and the pasture is infected more or less permanently, so that animals cannot graze on it without the risk of infection. Crows and foxes may also serve to disseminate the disease by feeding on infected materials and discharging the spores in their excreta. If the animal's body is deeply buried unopened, the bacilli cannot sporulate in the anaerobic environment to which the burying-process subjects them. The vegetative forms rapidly degenerate and die in buried carcasses. Pasteur suggested that earthworms might bring spores to the surface in their casts from the buried carcasses of infected animals and may help to spread the disease; but the danger from this source is practically very little. When a post mortem examination has been performed, there is a great danger of spreading the disease from the dead body.

Man:—The human being occupies an intermediate position between the highly susceptible animals (cattle and sheep) and the relatively immune animals (dogs, cats and birds). Man seems to be relatively insusceptible to anthrax infection. The disease is always communicated to him from animals and is the result of handling infected carcasses, skins or wools. The disease is met with in the following persons:—butchers, veterinary surgeons, shepherds, those who sort wools or hairs and those who work on or carry hides, e.g., glove makers, tanners, porters, blanket makers, etc. Infection has also resulted from the use of unsterile or improperly sterilised shaving brushes and tooth brushes, the bristles of which have come from infected animals.

The disease manifests itself in various clinical types which usually terminate in septicæmia. The clinical types are:—

1. *Cutaneous anthrax*—It is also called malignant pustule or hide-porter's disease or charbon.

2. *Pulmonary anthrax* or wool-sorter's disease.

These two are the main types.

3. *Intestinal anthrax*—a rare form.

Cutaneous anthrax *(Hide-porter's disease, Malignant pustule or charbon)*—The infection usually occurs through some small abrasions or scratches through which the spores gain entrance. The seat of inoculation is usually some exposed part of the body, such as the face, hands, forearms and the back of the neck and shoulders (a common place for hide-porters).

Within 12 to 24 hours after inoculation, a small red painful furuncle appears, which resembles at first an ordinary small pimple. This soon becomes vesicular, containing blood-stained fluid and is rapidly surrounded by an area of intense congestion. Sooner or later, the centre becomes necrotic and black in colour, surrounded by an angry, red, œdematous area, leading to the formation of the malignant pustule proper (*charbon*, a live coal). The typical picture of a malignant pustule (Fig. 210) appears as follows:—

(i) a black eschar of irregular shape in the centre, surrounded by

(ii) a ring of vesicles, this in turn being surrounded by

(iii) a congested and œdematous area.

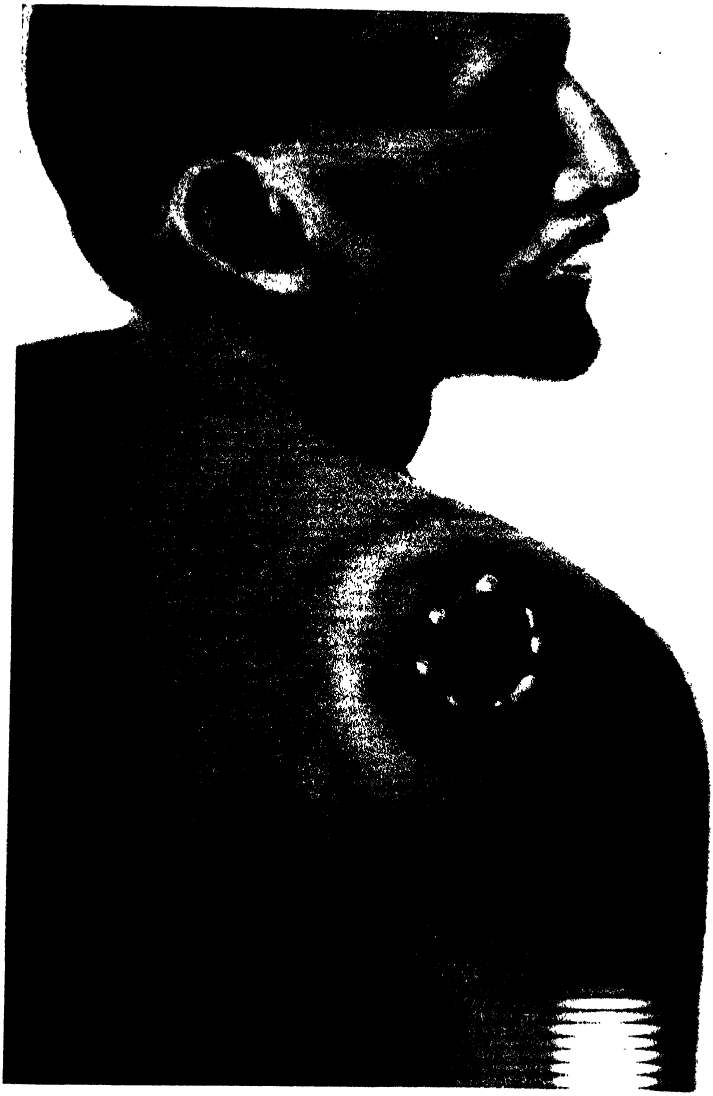


Fig. 210.

A typical picture of a case of *maligna...*

The bacilli are found at the periphery of the eschar and also in the ring of vesicles.

Along with the formation of the malignant pustule, there is a widespread œdema of the part with the involvement of the proximal lymph nodes; there is usually fever with general malaise.

In favourable cases, the lesion undergoes healing, the eschar becomes a scab, the inflammation subsides and recovery takes place. In other cases, the pathological process spreads further, till invasion of the blood stream occurs. Once a definite septicæmia has been established, death is the rule.

Pulmonary anthrax (*Wool-sorter's disease*)—Infection occurs through inhalation of spores contaminating dust, or threads of wool, hair or bristles of animals dead of the disease. These materials were contaminated with the discharges containing the bacilli which have undergone sporulation. Symptoms resemble those of pneumonia. The parts primarily affected are the lower part of the trachea and the large bronchi. The mucous membranes become swollen and intensely congested; there are often hæmorrhages. These ultimately break down and form small ulcers. The tissues around them become much œdematous and congested. The neighbouring lymph nodes, mediastinal and bronchial, are enormously enlarged and congested. Lungs show congestion, œdema and collapse. Pleural and pericardial effusions are often present. A state of septicæmia generally precedes death.

Intestinal anthrax:—The mode of infection in this form is by the alimentary route and occurs through the ingestion of infected meat and food. The usual heat employed for cooking or even canning does not necessarily kill anthrax spores. This form of infection is rare, but when it occurs, death is the rule. The clinical picture which follows is that of a violent hæmorrhagic enteritis with hæmorrhagic stools and great prostration.

Note:—Cases have been reported in which a hæmorrhagic **cerebrospinal meningitis** has occurred as a complication in various primary lesions.

Mode of action of B. anthracis:—The precise cause of death in anthrax is still uncertain. It is possible that mechanical means, such as capillary obstruction, may be a factor, but this cannot explain the whole picture which follows a course resembling that of toxæmia of great intensity. But neither a true exotoxin nor an endotoxin has ever been satisfactorily demonstrated for anthrax bacillus. Hence, the exact mode of action of B. anthracis cannot be stated definitely with our present knowledge.

THERAPEUTIC CONSIDERATIONS

Vaccines:—This is largely used as a method of prophylaxis for sheep and cattle. It was found that dead cultures were useless in producing an immunity in animals, whereas even minute quantities of virulent strains will produce death in susceptible animals. This led Pasteur to take resort to attenuated cultures for the production of active immunity in animals.

Pasteur's method of producing active immunity in sheep and cattle consists in the use of two strains of living cultures of varying degrees of attenuation, which is accomplished by a prolonged cultivation of the bacillus at a temperature of 42°C. in broth.

No. I vaccine, called "*Premier*" *vaccine*, is a weak one. The attenuation of this strain is brought about by growing an originally virulent strain at 42°C. for 24 days. This attenuated culture is found to be non-virulent to guinea-pigs or rabbits, but potent against still more susceptible animals, such as mice.

No. II vaccine, called "*Deuxieme*" *vaccine*, is a stronger one. Attenuation is brought about by growing the virulent strain at a temperature of 42°C. for 12 days. Such an attenuated strain is found to be virulent to guinea-pigs and mice, but not to rabbits.

Forty-eight hours' broth cultures of these attenuated strains are employed, using the No. I (weak) vaccine first, followed later by No. II (stronger) vaccine. The injections are given subcutaneously at an interval of 12 days. The dose for "Premier" vaccine for cattle is 0·25 c.c. and for sheep about half the quantity.

The immunity developed lasts for about a year. This method of prophylactic inoculation in cattle and sheep has reduced the mortality to a great extent. Before the use of this vaccine, there was a high mortality amongst cattle (5 per cent.) and sheep (10 per cent.) in France, but with its introduction, the figure has come down to 1 per cent.

Antivirus:—Besredka put forward his hypothesis on definite experimental grounds, that the only route of infection in animals is the cutaneous one and unless the latter is infected, the inoculated animal will not suffer from the disease. According to him, the cutaneous receptor cells are all-important in determining the infection. These cells can be effectively immunised by suitable means and thus the animal can be solidly protected against anthrax. This specific local immunity may be obtained by applying first an attenuated and subsequently a moderately virulent, broth culture on the shaven abdominal skin of a susceptible animal. In this way, very marked protection is conferred to the animal. Such cultures are called anthrax antivirus. Animals which are protected in this way do not develop any antibody in their blood.

Serum:—This is a variety of curative serum containing antibacterial substances and is extensively used therapeutically with success. There are many varieties of such curative sera in the market, obtained from horses, sheep and oxen, but Sclavo prepared the serum from donkeys **(Sclavo's Serum).** In an early case of malignant pustule, 2 [illegible] injections of 20 to 30 c.c. of the serum subcutaneously or intramuscu[illegible] will cause the pustule to localise and heal up rapidly. In an advanc[illegible], however, bigger doses and frequent administrations by intravenou[illegible], are indicated,

LABORATORY DIAGNOSIS OF ANTHRAX

This may be considered under two heads—antemortem and post mort.

Antemortem diagnosis, i.e., during the course of illness: diagnosis depends on the finding of the causative organism and its isolation in pure culture by cultural and animal inoculation methods. *In a case of malignant pustule, the best material for examination is a little serum from a vesicle. If no vesicle is obtained, the lesion should be gently scraped and serum allowed to exude. The latter contains many bacilli and may be collected for examination. In pulmonary anthrax, however, the organism should be sought for in the sputum. In late stages of both the cases, blood culture may yield a positive result, for which large quantities, such as 20 c.c., of blood should be taken.*

Methods:—

I. *Staining methods*—The films made from such a material may be stained with methylene blue and by Gram's method and the characteristic morphology studied. It should be remembered that the chains are never long, and may consist of only 2 to 3 bacilli.

II. *Cultural methods*—A loopful of the material should be inoculated into nutrient agar and broth media and the growth characters may be studied after 24 hours' incubation. The spore formation, the character of the growth in a gelatin medium and motility are important points for observation.

III. *Animal inoculation methods*—The examination should not be considered complete unless the material is inoculated into a susceptible laboratory animal. The inoculated animal will die of septicæmia in 3 to 4 days' time and the organisms can be identified in the smears from spleen or liver.

Commonest contamination and how to eliminate it:—The common saprophytic organism with which B. anthracis is likely to be confused is B. subtilis. The latter, as has already been stated, is frequently present in the dust, air and decomposing vegetable and animal matters and hence, any skin lesion is liable to contain it as a secondary contaminating organism. In this way, an ordinary furuncle or ulcer, caused by the common pyogenic cocci, may be wrongly diagnosed as a case of malignant pustule. Under such circumstances, mere morphological features and staining reactions may not be conclusive and the only reliable method to overcome the difficulty will be the pathogenicity test. B. subtilis, when inoculated into a guinea-pig, will not produce any fatal result.

How to send materials to the laboratory from a distant place:—A word may be said here about the method of sending materials for examination from distant places where requisite laboratory help is not available. A very simple method is to soak a few sterile silk threads in the serous exudate and after they are dry, they may be put into a test tube and sent to the laboratory. Another method is to put a few drops of the material on a clean slide or earthenware and allowed to dry on it after which it may be carefully packed and sent over to the laboratory. In this way, although the vegetative bacilli may die, spores will form and survive during the transit. In the laboratory, this dried silk or material on the glass slide or earthenware should be transferred to nutrient broth where the spores will germinate and multiply.

Post mortem diagnosis, i.e., after the animal is dead of the disease:—

I. **Demonstration and isolation of the causative organism** (for methods *vide supra*)—The best material for this purpose is obtained from the spleen. Inoculation into mice or guinea-pigs must always be done to confirm the diagnosis. The same procedure is recommended when the material is to be sent over to a distant laboratory for bacteriological diagnosis, using spleen juice, blood from a vein or haemorrhagic exudate in the lymph node or even a piece from the ear.

II. **Ascoli's thermo-precipitin reaction**—This is very useful in the post mortem diagnosis of anthrax. The principle and technique of the test have already been described on p. 366.

Disinfection

When an animal has died of anthrax or is suspected to have died of such an infection, it is always safe not to hold any post mortem examination on the carcass. A little blood or spleen or gland juice or a piece from the ear may be taken for bacteriological diagnosis and the entire carcass disposed of in such a way that the infection may not spread. The best method of disposal is to burn it. If that is not possible, the entire carcass may be buried deep in a pit with plenty of quick lime all round the body.

It is also important to disinfect carefully all fodder which may have been contaminated with the discharges from the nostrils and anus of the diseased animal. They should all be burnt away. The place where the animal is stalled, should also be thoroughly disinfected, either with bleaching powder solution or strong bichloride of mercury. The walls of the stall should be lime washed.

When a surgeon operates on a case of anthrax infection in man, all contaminated articles, such as dressings, instruments, bowls, etc., should be discarded and burnt or thoroughly disinfected. The case should be segregated at a safe distance from other surgical cases, for fear of spread of the infection. A separate set of instruments and dressing equipments should be provided for such a case and the wound should be handled only with gloved hands. After dressing, the gloves and instruments should be boiled for half an hour and kept in a strong solution of bichloride of mercury or lysol.

Cheap and improperly sterilised shaving brushes sometimes act as sources of infection and such articles should be properly disinfected before being used. This may be effectively done by soaking them in a strong solution of formaldehyde, bichloride of mercury or lysol for a long time.

PSEUDO-ANTHRAX BACILLI

These are aerobic spore-bearing organisms which are liable to be confused with the pathogenic member of the group [illegible] anthracis. For the differentiation, a table is appended on page 374. Th[illegible] organisms are saprophytes and represent a large number of species, t[illegible] important ones having already been mentioned on p. 361. They are ubiquitous in distribution, being found in milk, water, soil and dust, and are thus met with as contaminating organisms of culture media. For the consideration of these organisms in detail, the reader should consult higher works.

	B. anthracis.	**Pseudo-anthrax bacilli.**
Morphological:—		
Motility		Contains about 4 to 12 peritrichous flagella.
Capsule		
Chain formation	Very well marked.	Not well marked.
Cultural characters:—		
Broth ...	No turbidity; flocculent mass at the bottom.	Uniform turbidity.
Gelatin stab culture	Inverted fir-tree like growth; slight liquefaction at the upper part only.	Fir-tree growth is not typical; marked liquefaction.
Serological:—		
Ascoli's thermo-precipitin reaction		Doubtful; positive only in a very low titre.
Pathogenicity:—		
Guinea-pig	+ +	

o = Nil; + = Positive.

CHAPTER XXVI

SPORE-BEARING ANAEROBES

(CLOSTRIDIA)

Definition:—Anaerobes are organisms which are unable to grow in the presence of oxygen and the absence of which forms an essential condition for their cultivation. All these organisms possess endospores which are situated either at the centre (equatorial) or at the ends (terminal) or near the ends (subterminal). Another characteristic feature of this group of organisms is that the spores are always wider than the vegetative bodies, hence they are called "Clostridium" forms.

Classification of the Clostridia:—**A.** These organisms are usually classified from the nature and position of their spores and their action on proteins and carbohydrates. According to the latter property, they may be divided into two main groups as follows:—

Proteolytic

1. Clostridium tetani—Spherical and terminal spore.
botulinum
sporogenes — Oval and subterminal
4. ,, histolyticum
5. ,, aerofœtidum

Saccharolytic

1. Clostridium welchii
2. ,, septique
3. ,, œdematiens — Oval and subterminal spores.
4. ,, fallax
5. ,, chauvœi
6. ,, tertium—Oval and terminal spore.
7. ,, tetanomorphum — Spherical and terminal spores.
8. ,, sphenoides

This division merely signifies that the members of a particular group show a special action for that particular substance and it should not be applied too rigidly, as some of the saccharolytic group may have a proteolytic action and vice versa.

B. The following is, however, a list of the organisms based on their pathogenic properties:—

I. Organisms causing **tetanus** in man and animal—*Cl. tetani.*

Allied non-pathogenic organisms—*Cl. tetanomorphum, Cl. sphenoides, Cl. tertium, Cl. cochlearium.*

II. Organism causing **botulism** (food poisoning)—*Cl. botulinum.*

III. Organisms related to **gas gangrene**—

(a) Definitely pathogenic—*Cl. welchii, Cl. œdematiens, Cl. septique.*

(b) Less pathogenic—*Cl. fallax, Cl. histolyticum.*

(c) Not pathogenic by themselves—*Cl. sporogenes, Cl. aerofœtidum, Cl. bifermentans.*

IV. Organism causing **"quarter evil"** (black leg or symptomatic anthrax) in cattle—*Cl. chauvœi.*

Habitat:—They are widely distributed in nature where they exist as saprophytes. The primary habitat is the soil from which they are ingested by men and animals in whose intestinal canals they establish either a temporary or a permanent abode. For instance, Cl. welchii is always present in the human fæces and Cl. tetani in the cow and horse dung. The spores are always present and remain alive in the soil, specially when the latter is contaminated with human and animal excreta. Highly manured agricultural areas, garden soil, thoroughfares of large cities where animal-drawn vehicles are numerous and similar other places are particularly rich in these bacteria and their spores.

Morphology:—

Size and shape—These organisms are very pleomorphic. They are usually large, rod-shaped bacilli, varying in length from 3 to 8 μ.

Spore formation—It has already been mentioned that endospore formation is very characteristic of this species. These spores are always wider than the vegetative bacilli and, by their position in the bacterial body, they confer a characteristic appearance to the organisms.

(i) When the spores are situated at the centre (equatorial), the bacilli become spindle shaped, e.g., Cl. bifermentans.

(ii) When the spores are subterminal, the bacilli become club shaped, e.g., Cl. botulinum, Cl. sporogenes, Cl. welchii.

(iii) When the spores are oval and terminal, the bacteria assume a "tennis-racket" form, e.g., Cl. tertium.

(iv) When the spores are spherical and terminal, the bacteria assume a "drum-stick" form. e.g., Cl. tetani.

All the pathogenic members of this group can form spores in the animal body (Cf. B. anthracis, which do not form spores in the animal body).

Flagella and motility:—Nearly all the members of this group are motile and possess numerous peritrichate flagella, with the exception of Cl. welchii, Cl. bifermentans and Cl. tertium. The motility of these organisms is somewhat slow and sluggish, as compared with the very rapid movement of Eberthella typhi.

Capsule formation:—Only Cl. welchii possesses a well marked capsule which is most marked inside the animal body. Others have no capsule.

Staining reactions:—All the organisms of this group are Gram-positive. They may also be stained with other aniline dyes. Special stains are necessary to demonstrate the flagella, the spores and the capsules (vide chapter II).

Cultural characters:—They can be cultivated both in liquid as well as in solid media, but for isolation, the latter are necessary. The following media are suitable for their cultivation:—

(i) *Glucose agar.*

(ii) *Coagulated serum and egg*—These media are particularly used to test the liquefying action of the proteolytic group.

(iii) *Blood agar.*

(iv) *Cooked meat medium*—The organisms which grow in this medium will either digest the meat and turn it black (proteolytic group) or turn it pink without any digestion (saccharolytic group).

NOTE:—Colonial characters are best studied in surface cultures, shake cultures or stab cultures.

Character of the colonies:—The anaerobic bacteria, when growing on the surface, produce colonies which vary widely in their characters. For a novice, it is difficult to distinguish these organisms by these characters alone, but in the hands of an experienced worker, the colony formation constitutes a very important method of differentiation. For those who would like to study this subject, the following points would be undoubtedly very helpful in the study of the colonial characters of the important organisms associated with gas gangrene. A magnifying glass is essential for this study.

In the case of organisms of gas gangrene:—

(i) Colonies which have a round or slightly wavy margin and which remain superficial without any tendency to penetrate deeper into the substance of the medium.

When they are opaque, they are *Cl. welchii.*
When they are translucent, they are *Cl. tertium.*

(ii) If the crenations of a somewhat round colony are very much exaggerated, so that a fern-like appearance is produced, it is very suggestive of *Cl. septique.*

(iii) When the growth is somewhat wooly and tangled, with processes dipping into the substance of the agar, like the roots of a plant, it is very suggestive of *Cl. sporogenes.*

In the case of the *Cl. tetani*, the growth tends to spread over the whole surface as a thin layer with irregular edges, resembling the growth of Proteus vulgaris (B. proteus).

The growth of *Cl. botulinum*—The surface colonies are large, transparent and filamentous with a granular surface and [illegible] edge.

Metabolism:—

1. They require an anaerobic condition for their growth.

NOTE:—These organisms, however, like any other living things, require oxygen for their life and metabolic activity. This they obtain by breaking up the complex constituents of the medium and utilising the oxygen which is liberated by such chemical decomposition, instead of directly obtaining it from the atmosphere.

2. They produce strong hæmolysins and therefore hæmolysis is well marked in blood agar medium.

3. During their growth, they produce much gas, even in media which are free from all fermentable carbohydrates. The gas consists of CO_2, H and H_2S in varying proportions, according to the nature of the organism and the medium used.

4. In the case of the saccharolytic group, there is production of much organic acid (butyric acid), whereas in that of the proteolytic group, ammonia in large quantities is evolved. The relative importance of these decomposition products will be discussed later (*vide infra*).

Biochemical reactions:—

Proteolysis—The substances used for the study of the proteolytic action are peptone, gelatin, casein (milk), serum, egg-white and fibrin. The organisms which possess a slight proteolytic action cannot attack the higher proteins, such as fibrin, egg-white and serum.

Saccharolysis—Litmus milk and various sugars are used for this purpose. In litmus milk, however, the saccharolytic group produces acid and varying amounts of gas with clot formation, whereas the proteolytic group digests the milk with the production of an alkaline reaction.

Resistance:—All the members of this group are very resistant to drying, heat and disinfectants. For instance:—

(a) The spores of Cl. tetani may remain alive in the dried earth for many years.

(b) The spores of Cl. botulinum withstand boiling for 3 to 4 hours.

(c) The spores of Cl. sporogenes may survive exposure to a 5 per cent. carbolic acid solution for more than a week.

Toxin production:—All the members of this group produce very powerful soluble extracellular toxins which, on injection into susceptible animals, give rise to the corresponding antitoxins, capable of being neutralised by the toxins. The latter may have, in some cases, a specific action on certain tissues, e.g., botulinus toxin on parasympathetic system, tetanus toxin on the central nervous system, and so on. In addition to this, they produce powerful aggressins which help them to flourish in the infected tissues (*vide* pp. 39, 103). Some of them also produce powerful hæmolysins.

Serological reactions:—Agglutinating sera have been prepared against the various members of the anaerobes, and these have been utilised in subdividing a particular species into several strains. It is to be noted that, like the coli-typhoid group of organisms, the motile species of the anaerobes contain both the thermolabile H or flagellar antigens and thermostable O or somatic antigens.

As most of these organisms possess a powerful exotoxin, antitoxic sera have been prepared against them and particularly against Cl. tetani, Cl. botulinum, Cl. welchii and some others. These are employed as therapeutic and prophylactic measures with much success.

Pathogenicity:—The production of the pathogenic effects depends entirely on their toxin production. Under ordinary circumstances, these organisms are probably unable to grow in the healthy tissues which are directly exposed to the air. There must be available some suitable condition for their growth, multiplication and toxin-production; the latter is then absorbed into the system and produces the pathogenic effects. Such a condition may be obtained in lacerated and infected wounds which result in a large amount of tissue necrosis. When such dead and necrotic tissues provide a condition of anaerobiosis, the bacteria find a suitable nidus for growth and manufacture of toxin in the local area. Thus, it will be seen that the effect of an anaerobic infection is a typical example of intoxication (*sapræmia*).

It requires a little explanation as to the reason why dead, necrotic and infected material favours the growth and multiplication of these bacteria. During the course of an infection in a lacerated wound, many common pyogenic bacteria are implanted along with the spores of the anaerobic organisms. At the beginning, the former rapidly multiply and in their growth, they use up a very considerable amount of oxygen. Besides, they also help in the necrosis of the lacerated tissues. Thus, the anaerobes get a chance to grow in this necrosed tissue under a condition of diminished oxygen supply. Another very important factor introduced in such a condition is the effect of the toxins of some of the anaerobes, e.g., Cl. welchii and Cl. septique. The extracellular toxins of these bacilli, when present in the wound even in very low concentrations, prevent, by their aggressins, the approach of the phagocytic cells and thus allow the organisms to multiply and grow without any check. Although the main effect is due to the extracellular toxins which are absorbed from the infected wound, there is always some tendency for the organisms to penetrate and invade the deeper tissues of the host, specially towards the end. In experimental animals, the pathogenic anaerobes invade the internal organs very rapidly and may be recovered from their blood. But in the case of the average human infection, they remain mainly localised in the wound and, only in fulminating cases, may spread rapidly into distant areas and occasionally invade the general blood stream. The important diseases caused by the various pathogenic anaerobes in man are ***tetanus***, *botulism* and *gas gangrene*.

ANAEROBIC METHODS

It must be remembered that all the ordinary culture media contain some oxygen after their preparation and, when left in co[illegible] with the air, they will absorb more from the atmosphere. Hence, it [illegible]essary to devise special methods to make the culture media free from oxygen. This is carried out as follows:—

1. *Vacuum.*
2. *Displacement of oxygen (Bulloch's jar).*
3. *Absorption of oxygen—chemical means (Buchner's tube), combustion (McIntosh and Fildes' jar) and biological means.*
4. *Reduction of oxygen (deep glucose agar, Noguchi's medium, Robertson's cooked meat medium).*

1. ***Vacuum:***—During the early days, anaerobic conditions were obtained by means of an ordinary vacuum pump. The culture tubes were kept inside a bell jar, the rim of which was hermetically sealed and from which all the air was pumped out. Although it served the purpose of the pioneer workers, it was soon found that a perfect vacuum could not be obtained. Hence, this method was replaced by others.

2. ***Displacement of oxygen:***—In this method, the oxygen in the culture tube is expelled by passing an inert gas for a prolonged period, till all the oxygen is completely replaced by the latter, so that the culture medium is now surrounded by an atmosphere of the inert gas. In common practice, hydrogen gas is used and is obtained from a Kipp's apparatus. A simple device is found in what is known as **Bulloch's jar.** (Fig. 211).

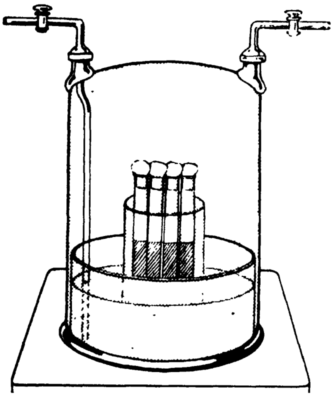


Fig. 211.
Bulloch's anaerobic apparatus.

3. ***Absorption of oxygen:***--This may be obtained in three ways:—(a) Chemical means, (b) combustion method and (c) biological or bacterial means.

Chemical means—In this process, such chemical reagents are employed as are capable of absorbing large quantities of oxygen. Usually, the chemicals employed are caustic soda and pyrogallic acid. When the two substances are mixed together, an alkaline solution of pyrogallol is produced which has the remarkable property of absorbing large quantities of free oxygen. At first, such a solution is of a light straw colour, but later becomes dark brown as it absorbs oxygen. *Buchner,* in 1888, first used this principle which is still used in our laboratories and the device is known as **Buchner's tube.** It is a stout walled large test tube, 8″ × 1″, the lower end of which is blown into a bulb. Above the bulb is a ledge over which the inoculated culture medium rests. The tube is furnished with a well fitting rubber stopper. For use, some pyrogallic acid is placed in the bulb and strong caustic soda (20 per cent.) is added, so as to occupy half of the bulb. This forms an alkaline pyrogallol. The inoculated medium is then quickly introduced and the large test tube is closed tightly with the rubber stopper and finally sealed with melted paraffin (Fig. 212). The apparatus is then put in the incubator.

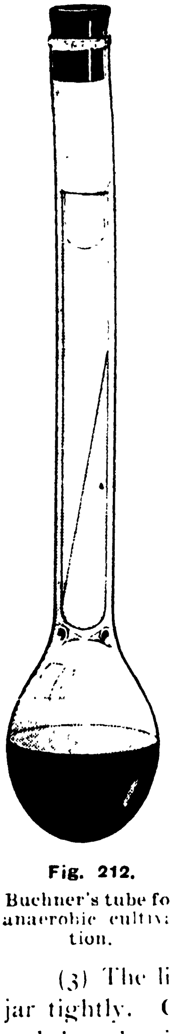


Fig. 212.
Buchner's tube for anaerobic cultivation.

Combustion method—The principle of this method depends on the removal of oxygen by the oxidation of hydrogen and this is conveniently carried out in the **McIntosh and Fildes' Jar** (Fig. 213). The apparatus is made of a stout glass jar of 8" × 5" (more recently gun metal and aluminium jars are also used), the rim of which is flanged so as to fit a clamp to fix a metal cover over it. The lid is furnished with two needle valves which act as inlet and outlet for gases. From the undersurface of the lid is suspended a metal spool of palladinised asbestos wound round a fine coil of resistance wire, the two ends of which are connected with two terminals fitted on the lid. When an electric current is passed through the wire, the spongy palladium becomes heated and acts as a catalytic agent, causing slow combustion of hydrogen and oxygen to form water. The whole of the spool is surrounded by a wire gauze to prevent a sudden explosion.

How to use the apparatus:—

(1) The opposing surfaces of the lid and jar are cleaned with xylol or benzol and smeared with vaseline.

(2) The inoculated test tubes and Petri dishes are placed inside, with some cotton wool or lint at the bottom. Along with this, an indicator tube or a tube of glucose broth containing a few drops of alkaline methylene blue is introduced. This indicator becomes colourless when the anaerobiosis is complete.

(3) The lid is then clamped down on to the jar tightly. One of the needle valves is closed and the other is connected with a suction apparatus to create a vacuum inside the jar. When this is obtained, the valve is closed.

(4) Hydrogen is now passed from a Kipp's apparatus through one of the valves as arranged in Figure 214. The inclusion of a mercury manometer is to record the degree of pressure in the system.

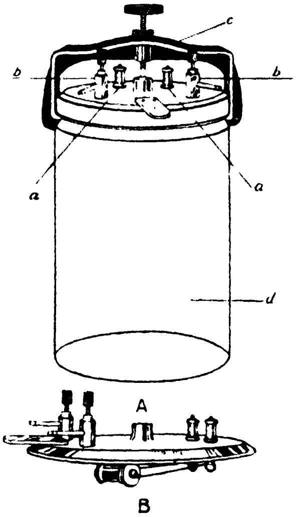


Fig. 213.
McIntosh and Fildes' anaerobic jar.
A—The jar as a whole. a,a'—terminals; b,b'—needle valves; c—metal clamp; d—glass jar.
B—Metal cover, showing how the spool has been suspended from the under surface of the lid.

(5) Both the valves are now closed tightly and the terminals are attached to an electric supply through a resistance. A curr[illegible] 200 volts (two 16 c.p. and one 8 c.p. carbon lamps in parallel) w[illegible]nce for ordinary purposes.

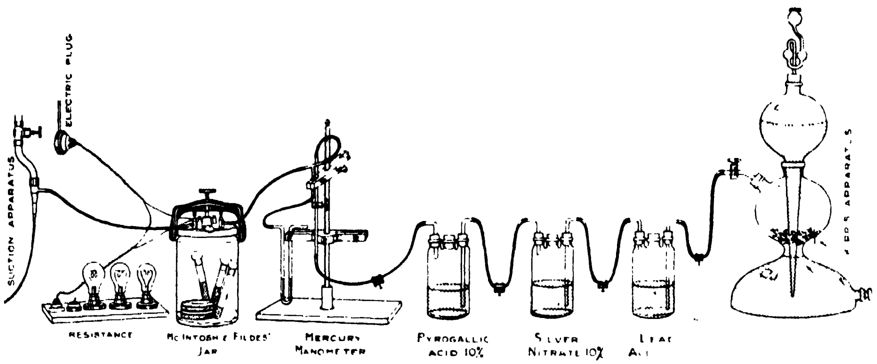


Fig. 214.

Use of McIntosh and Fildes' jar for anaerobic cultivation. The hydrogen evolved from Kipp's apparatus by the action of zinc and sulphuric acid is passed through a series of wash bottles to remove sulphuretted hydrogen, arseniuretted hydrogen and oxygen respectively.

(6) In a short time, dew drops will appear on the walls of the jar. A negative pressure inside the jar is produced, as indicated by the manometer. More hydrogen is introduced through the valve. The current is passed again and combustion allowed to go on.

This process is repeated several times till all the oxygen inside the jar is used up. This is indicated by the discharge of colour in the indicator tube and also by the manometer showing an equal pressure in both the limbs. The jar may now be disconnected from · the resistance and the valves tightly closed. It is now ready to be placed inside the incubator.

Biological or bacterial means:—When two different kinds of bacteria are allowed to grow in a confined space, one of them may use up so much oxygen for its growth that there is very little left in that space for the growth of the other variety. Hence, anaerobic condition is established. Serratia marcescens (B. prodigiosus) is one such organism which, while actively growing in a confined space, readily removes the oxygen from the contained air. In the same plate, which is carefully sealed with paraffin wax, S. marcescens (B. prodigiosus) and the required anaerobic organism are grown together in separate halves. Serratia marcescens (B. prodigiosus) during growth absorbs the oxygen for itself and creates anaerobiosis.

4. ***Reduction of oxygen:***—This is done by using a medium containing some reducing agent. The commonest reducing body is **glucose** which is added in about 2 per cent. strength. The method is very convenient for solid media. The inoculation may be made either as a *shake culture* or as a *deep stab culture* in which the upper part of the medium is melted and allowed to solidify so as to seal the needle track in the medium.

Another very good reducing body is **freshly excised animal tissue,** such as rabbit's kidney, spleen, testicle or heart. By adding portions of such a tissue in the medium which is usually liquid and preventing further access of oxygen, a very efficient anaerobic condition is established. Such a method of cultivation was extensively employed by **Noguchi** for the culture of spirochætal organisms. The disappearance of oxygen is considered to be due to auto-oxidation of the unsaturated fatty acids contained in the meat. A piece of freshly excised tissue, preferably the kidney, removed under strict aseptic conditions, is placed at the bottom of a high test tube containing the culture fluid which may then be inoculated. Sterile melted paraffin or vaseline is then added, producing a layer on the top and protecting the subjacent medium from external atmospheric oxygen. Instead of fresh tissues, heated tissues act in the same way, as in **Robertson's cooked meat medium** which is prepared as follows.

Robertson's cooked meat medium (modified by *Martin and Lepper*)—

1. 500 gms. of fresh bullock's heart, freed from fat, are taken and minced finely.
2. An equal volume (500 c.c.) of boiling N/20 caustic soda is added and allowed to boil for 20 minutes. The mixture is then allowed to cool in running water.
3. The mixture is then strained through a muslin gauze and the minced meat is then spread out on the cloth and allowed to dry partially.
4. The meat is then placed in test tubes to a depth of 2 inches and is covered with nutrient broth (pH 7·5), until it stands 1 inch above the meat.
5. The tubes are then placed in a bath of boiling water for half an hour to drive off the entangled air.
6. The tubes are sterilised in the autoclave at 120°C. for 20 minutes.

After inoculation, the cotton wool plug may be sealed with melted paraffin to prevent further access of air. The exclusion of air may be further ensured by putting the inoculated medium inside the McIntosh and Fildes' jar, thus ensuring a double safeguard.

Certain **other reducing substances,** such as cysteine, glutathione and succinic acid, when added to a medium, produce an anaerobic condition, thus providing a means of cultivating anaerobic bacteria under aerobic conditions. Thus, when a minute amount of cysteine hydrochloride is added to nutrient broth or agar, it parts with its hydrogen in the medium and becomes cystine. The liberated hydrogen absorbs the oxygen in the medium, producing a condition of what is called reduction potential.

In the preparation of the media, cysteine hydrochloride is added to nutrient broth or melted agar at 80°C., in a concentration of 0·3 per cent. The medium becomes turbid and also acid. Its reaction is then adjusted to pH 7·4 by titrating with a 10 per cent. NaOH solution. It is finally sterilised in the steam steriliser for half an hour. Inoculation into agar should be made in the melted condition (at 50°C.) and the latter is then poured into plates. The growth is detected at the edge of the m[illegible].

Note:—When reduction of oxygen is used as a m[illegible] anerobiosis, it is always advisable to use either a Bulloch's [illegible] a McIntosh and Fildes' jar.

CLOSTRIDIUM TETANI

(Bacillus tetani)

The causative organism of tetanus.

HISTORICAL:—Two Italian workers, *Carle* and *Rattone*, first described their experimental observations on tetanus. *Nicolaier*, in 1884, first discovered the organisms by injecting samples of earth into laboratory animals. *Kitasato*, in 1889, isolated the bacillus in pure culture. In 1890, *von Behring* and *Kitasato* discovered the antitoxin.

HABITAT:—The organism is commonly encountered as a saprophyte in the intestines of some of the domestic animals, such as the horse, cattle and sheep. It is also found in the intestines of man, which may explain the occurrence of cryptogenic tetanus. The spores of the organism are present in the superficial layers of the soil and they are particularly frequent in cultivated and manured soil.

MORPHOLOGICAL CHARACTERS (Fig. 215):—It is a fairly long, thin, pleomorphic bacillus, about $5\ \mu$ in length. It possesses many peritrichate flagella and is motile. Spore formation takes place at one of the terminal ends, producing one spherical spore, and this gives the bacillus a *" drum stick "* appearance. In the usual stained preparation, such a spore is never found free but always attached to the bacillary body.

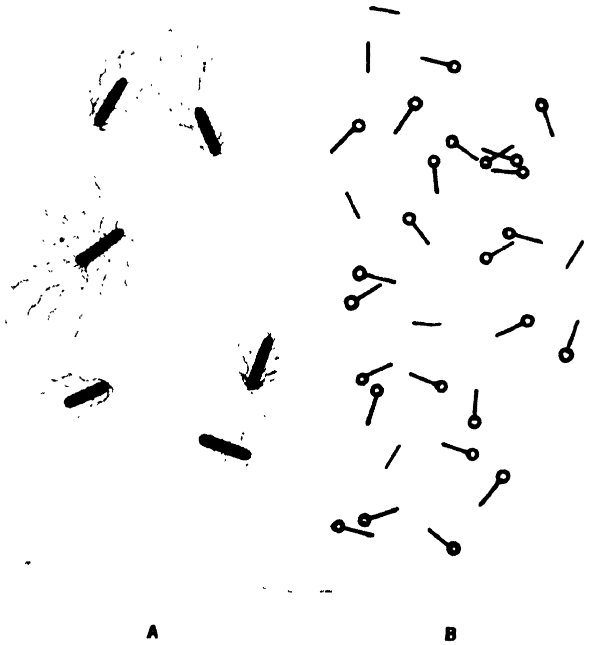


Fig. 215.
Morphology of *Clostridium tetani.*
A—peritrichate flagella; B—spherical and terminal spores (drum-stick form).

STAINING REACTIONS:—The bacillus is Gram-positive. It may be stained with other aniline dyes. Special stains are necessary for the demonstration of the spores and flagella.

CULTURAL CHARACTERS:—As usual with the anaerobic bacteria, the organism can be cultivated only under strict anaerobic conditions. Usually, incubation for 4 to 5 days is necessary to produce a sufficient growth.

Glucose agar:—The growth is usually shown by the production of gas and disruption of the medium. The *colonies* are, as a rule, small, somewhat opaque, with irregular, filamentous edges. The growth is usually more vigorous in the deeper parts and very scanty superficially.

If the inoculation is made in the water of condensation of a glucose agar slope, the growth tends to spread over the whole surface as a thin layer with irregular edges, resembling the growth of Proteus vulgaris (Bacillus proteus).

In *stab cultures*, the growth resembles the appearance of a fir-tree.

In ***nutrient broth*** with or without glucose, there is growth with uniform turbidity and a granular deposit at the bottom. A peculiar manure-like odour develops in the culture tubes.

The growth of Cl. tetani is very luxuriant in ***cooked meat medium.*** The broth becomes turbid and there is some evolution of gas but the meat does not show any alteration in colour in the way of blackening nor is it digested.

In blood agar:—There is growth with hæmolysis.

BIOCHEMICAL REACTIONS:—

Saccharolytic properties—Cl. tetani does not ferment any sugar.

Proteolytic properties—It *produces indol* in peptone water. In *litmus milk*, the colour of the litmus may be discharged without any other gross change (no coagulation). *Gelatin* is slowly liquefied. *Coagulated serum* is rendered more transparent and softened but is not liquefied.

VIABILITY:—Although the vegetative bacillary forms cannot resist heat, desiccation and the action of antiseptics more than the other common bacteria, the spores are very resistant, and may remain alive for many years. For instance, they can withstand boiling for 15 to 20 minutes and can stand the action of a 5 per cent. carbolic acid solution for hours. The viability of the spores is still further prolonged if they are kept in a moist humid place, away from direct sunlight. It is for this reason that they are present so abundantly in manured agricultural and garden soil.

SEROLOGICAL VARIATIONS:—Injection of [illegible]d organisms into a rabbit leads to the development of agglutinins. R[illegible]s of agglutination tests, *seven* serologically different varieties of Cl. t[illegible]ave been isolated. But the toxins produced by all these different typ[illegible]rganisms are the same and are neutralised by the antitoxin prepared [illegible] any one type.

TOXIN PRODUCTION:—Cl. tetani, during its growth, produces a toxin which diffuses out of the bacterial body into the surrounding medium. Hence, it is *an extracellular toxin*. All the pathogenic effects are due to this soluble exotoxin. The best medium for the study of tetanus toxin is a liquid one. In this, the bacteria are grown anaerobically and then this liquid culture is filtered through a porcelain filter when the bacteria-free filtrate is found to contain the toxin.

In the liquid state, tetanus toxin is very delicate and thermolabile and hence decomposes readily and soon becomes innocuous. But it retains its qualities intact if kept in a dried, powder form. It is in this form that the toxin is kept in commercial firms which undertake the work of immunisation and manufacture of antitoxic serum.

Tetanus toxin is one of the most powerful poisons known. It is so toxic that 0·00001 c.c. is enough to kill a mouse weighing 10 gms. The following are some of the important facts about tetanus toxin:—

(1) It consists of two distinct varieties of poison—(a) One kind of poison produces convulsions and is called *tetanospasmin*, and (b) the other variety has a marked hæmolytic effect and is called *tetanolysin*.

(2) When taken by the mouth, the toxin is completely destroyed and becomes harmless. When injected, it is absorbed through the motor end-plates in the muscles and carried along the motor nerves to reach the anterior horn cells in the spinal cord (Fig. 216). The toxins may reach the motor nerve cells in the brain either by extension from the spinal cord or through branches of the motor cranial nerves. Any way, not until the motor nerve cells of the brain and spinal cord are reached, do symptoms of tetanus appear.

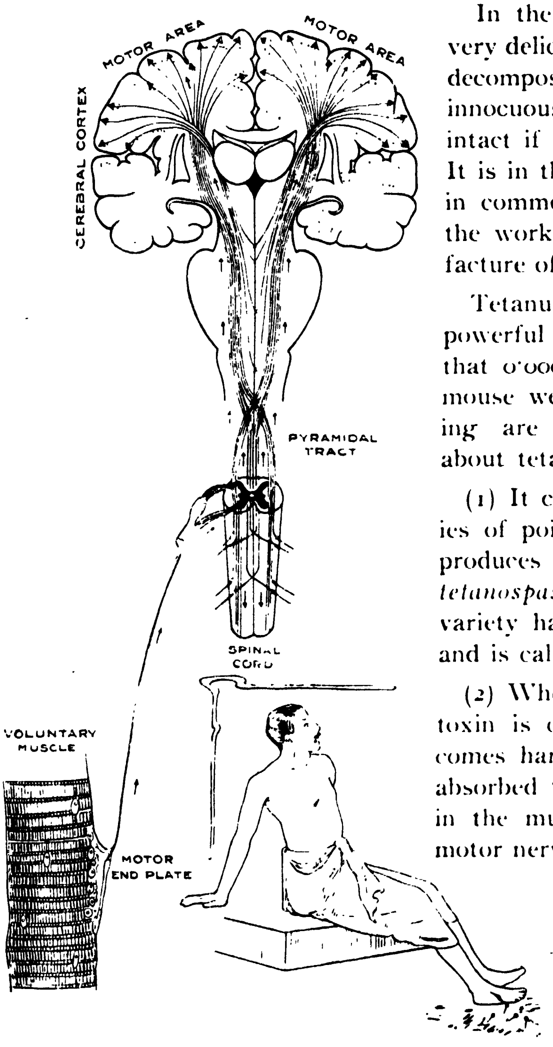


Fig. 216.

Mode of action of tetanus toxin.

Note the infection of the wound with spores of Cl. tetani, where they germinate and develop into vegetative forms. The bacilli multiply at the site of injury, liberating a large amount of exotoxin which is absorbed into the circulation and reaches the muscles. The toxin now enters the central nervous system through the motor end plates and motor nerves

(3) It has been supposed that the toxin, after reaching the motor nerve cells, enters into some sort of combination which, though not permanent, is still sufficiently fixed, so that subsequent action of the antitoxin is unable to dissociate it. Such union with nerve elements results in the enormous increase of tone and constant irritation, simulating the action of strychnine.

(4) Some time is always required for this combination and the development of tetanic symptoms. This is called the *incubation period* and it varies under different conditions, but the longer it is, the milder and less fatal is the attack. The incubation period in acute cases varies from 4 to 14 days and, on an average, it is 7 or 8 days. In chronic cases it may be about a month. The incubation period, however, is prolonged (45 days) when the patient recieves a prophylactic injection (500 units) of antitoxin.

PATHOGENICITY

The toxins of Cl. tetani are extremely harmful to men as well as to animals but their susceptibility in this respect varies a good deal. As a rule, human beings and horses are the most susceptible, while cats, dogs and birds, specially the domestic fowls, are highly resistant. Cold blooded animals are, as a rule, almost immune to tetanus toxins. Some of these animals are turtles, alligators and frogs. All the usual laboratory animals are affected by tetanus toxins.

In man and susceptible animals, the Cl. tetani produces the disease known as *tetanus*. The symptoms and signs are ushered in, at a very early stage, in the group of muscles in the immediate neighbourhood of the wound, by hyperexcitability and painful twitchings and it is only when the disease is fairly advanced that the face and jaw muscles show the characteristic spasms. Later on, the muscular rigidity and twitchings may spread to other muscles, producing stiffness of the limbs and body. The spasm of the facial, jaw and cervical muscles gives rise to " risus sardonicus," trismus (difficulty in opening the mouth) and stiff neck. Subsequently, the tonic contractions and spasms spread to all the muscles of the body, resulting in the picture of a fully developed case of tetanus.

Portals of entry:—Usually, there may be found the history of some trauma, of varying grades, in some part of the body. In some cases, injury to the soles of the feet by a splinter or a nail is very constant. Lacerated wounds, resulting from street accidents, are often important causes. Occasionally, the wound may be very trivial and completely forgotten by the patient and is detected only on careful questioning. Apart from the above causes, the following are worth remembering:—

(i) *Any suppurative lesion in the body* may be s[illegible]rily infected, such as middle ear disease, bed sores, abscesses and fur[illegible]cne pustules, and *in fact, any ulcerative condition in any part of the body. In this connection, it should be mentioned that smallpox vaccination may be followed by tetanus.*

(*ii*) Tetanus may sometimes follow hypodermic injections, particularly of drugs which are marked *tissue irritants*. *Quinine* heads the list in this respect and the development of tetanus after quinine injection is dreaded by physicians and surgeons in the tropics. The spores are thought to enter into the needle-track from outside and germinate and multiply in the necrosed tissue caused by the toxic effect of the drug.

(iii) The use of unsterilised and contaminated dressings on an open wound in the body may be attended with tetanus. Similarly, the use of insufficiently sterilised *catgut* (prepared from sheep's gut) in operations on the body may also be attended with tetanus.

(iv) In the past, when solutions of *gelatin* were injected to increase the coagulative power of the blood and also as a hæmostatic measure, tetanus not infrequently developed. But now-a-days, its use has almost been given up on account of this danger.

(v) Tetanus may affect the *parturient mother*, either during labour or more frequently, during the puerperium, specially if there has been improper handling and when instruments have been used. The spores are carried from outside into the uterine cavity where blood clots and bits of placenta form suitable nidus for the germination of the spores and their subsequent multiplication.

(vi) Tetanus may occur in the *new born* and the portal of entry in this case is the raw unbilical cord. This kind of tetanus is called "*tetanus neonatorum*." The infant mortality from this condition is fairly high in our country, although in European and American countries, it is almost insignificant. This difference appears to be due to the ignorance of the mass in our country regarding proper post-natal precautions, and the employment of unclean and illiterate country "*Dhais*" for confinement "*Dhais*" are low caste illiterate native women, who conduct labour cases amongst people in the villages of Bengal.

THERAPEUTIC CONSIDERATIONS

We have already seen that the signs and symptoms of clinical tetanus are due to the effect of a powerful extra-cellular toxin produced by Cl. tetani locally in the wound. We have also seen that this toxin, though absorbed into the circulation, cannot produce any harmful effect, unless and until it is taken up by the motor nerve endings, and that it travels along the nerves to reach the motor nerve cells of the brain and spinal cord, where a fairly firm combination takes place and that it is only after this process that symptoms are produced. All these processes will naturally require some time which, as we have said above, is known as the incubation period. Hence, the therapeutic effect which consists in the neutralisation of the poison, is obtained before the latter has had time to combine with and be fixed to the nerve cells. Once this combination has taken place, it becomes extremely difficult, even well nigh impossible, to dissociate the toxin to be neutralised by the corresponding antitoxin.

It has been definitely found by the study of a large series of cases during the Great War that prognosis of a case depends to a great extent on the incubation period. The earlier the onset of symptoms, the more likely is death to occur as will be shown from the table below.

Relation between incubation period and mortality:—

In unprotected cases (Permin's series)—

Incubation period	10 days or less	...	Mortality	90	per cent.
,, ,,	over 10 days	...	,,	70	,, ,,

In protected cases (Bruce)—

Incubation period	7 days or under	...	Mortality	58	per cent.
,, ,,	8 to 14 days	...	,,	49	,, ,,
,, ,,	15 to 21 days	...	,,	30	,, ,,

In actual treatment, the antitoxic serum is used as a *prophylactic* as well as a *curative agent*. When a case is diagnosed early, and the symptoms are mild, large doses of the serum, varying from 15,000 to 30,000 *units*, should be administered without delay. A part of this quantity should be injected around the neighbourhood of the wound, if there be any present at the time, and the rest should be given intramuscularly. This process may be repeated every 24 to 48 hours till improvement sets in. All the other ancillary therapeutic agents should be used along with the antitoxic serum.

In severe cases, the amount of serum and the route of administration should be somewhat different. In such a case, apart from injecting the serum around the neighbourhood of an existing wound, large doses should be injected intravenously. It is in such cases that intrathecal injection of serum is indicated. For this purpose, a preliminary injection of morphia is necessary. A lumbar puncture is first made and some cerebrospinal fluid is withdrawn and collected in a test tube. Through the lumbar puncture needle, an equal quantity of concentrated antitoxin, at body heat, is injected. The process may be repeated at regular but frequent intervals. In addition to the intrathecal injection, 20 to 30 thousand units should be given intravenously or intramuscularly. Like treatment of cases of diphtheria, it is always safer to inject more serum than what is thought to be necessary. In a case of average severity, the use of a total quantity of *100 [illegible] thousand units* is not considered to be too much.

Prophylaxis:—The greatest use of antitetanic serum is in prophylaxis and its introduction into general use has reduced both the incidence as well as the mortality from the disease. From a very accurate study of this subject during the Great War in 1914, it was found that after the introduction of prophylactic injections of antitetanic serum in wounded soldiers, the incidence fell from 9 per thousand to 1·4 per thousand and the mortality was lowered from 53 per cent. to about 22 per cent.

In the prophylactic use of the serum, the quantity to be administered should depend on the character and the extent of the injury and also on the time that has elapsed after receiving the injury. In civil practice, in a case of average severity, the injection of 1500 *units* is the rule. But if the amount of laceration is very severe and some time has elapsed, the injection of a much bigger dose is always necessary. It is also desirable to inject a part of the serum around the neighbourhood of the wound. Another very important matter in such badly lacetrated and grossly contaminated wounds is to combine the tetanus antitoxin with a sufficient dose of anti-gas-gangrene serum, as the toxins of the gas-gangrene-producing organisms always help the pathogenic effects of Cl. tetani. If considerable delay has occurred, larger doses of antitoxin are absolutely necessary. Another very important matter to remember in the prophylaxis against tetanus is the fact that all traces of antitoxin disappear from the blood within 10 to 14 days after its administration (*vide* p. 156). If at this time, the wound is still in such a condition that spores of Cl. tetani contained in it or infecting it can germinate subsequently and produce toxin, tetanus may result. Hence, under such conditions, it is always safer *to repeat the prophylactic dose of the antitoxin after an interval of 6 to 8 days till the wound heals up.* Of course, the necessity for a vigorous surgical treatment of such wounds to prevent germination of spores after the effect of the antitoxin has diminished, should always be remembered.

The question of **surgical prophylaxis** is a very important matter in the prevention of tetanus. All dirty and lacerated wounds should preferably be excised at the earliest opportunity, if possible. All splinters, dirty wood or metals, clothes and similar foreign bodies should be scrupulously removed from the wound which should be surgically cleansed, washed and satisfactorily drained. Unless this is done, the wound will produce a very favourable nidus for germination of the spores and growth of the anaerobic bacteria. In this connection, the use of a tourniquette for a long period and very tight plugging to effect hæmostasis are both undesirable, as they help to lower tissue resistance by interfering with nutrition.

LABORATORY DIAGNOSIS

The laboratory diagnosis of tetanus consists in discovering the Cl. tetani and is sometimes very difficult. In any case, as much of the suspected material as possible should be collected for examination. In cases of suppurative lesions, the pus or the discharge is suitable, whereas in cases of necrotic and ulcerated wounds, a portion should be excised or scrapings from the ulcer collected. Materials suspected to be contaminated with the spores should be taken in sufficient amounts, otherwise a negative result may be obtained.

Methods of diagnosis consist of the following:—

1. *Microscopical examination.*
2. *Cultural examination.*
3. *Animal inoculation.*

1. **Microscopical examination** of a direct smear is always advisable to see not only the tetanus-like bacilli, but also to know the nature of the bacterial contamination. Bacilli with round terminal spores should be looked for. Usually, their number is very scanty and it may require a very prolonged and patient search to find them.

2. **Cultural methods**:—This is the most important method. The best medium for this purpose is Robertson's cooked-meat medium. Strict anaerobic condition should be obtained. The medium, after inoculation, should be incubated for 4 to 5 days continuously. After this time, examination should be made for the typical bacilli.

For purposes of isolation, such a culture medium with the growth in it, should be heated to 75° to 80°C. for about 20 minutes to half an hour when all the vegetative forms of the bacilli will be killed but the spores will survive. It may then be plated and the characteristic colonies picked out. Another very good method is to inoculate the water of condensation of a sloped medium with the heated culture when, within 12 hours, a filmy, proteus-like growth will spread on the surface of the slope. Two or more similar subcultures should be made to obtain a pure culture. The organisms thus isolated should be studied as to their morphology, staining reactions, biochemical and other cultural characters. They may be finally tested against an agglutinating serum.

3. **Animal inoculation**:—Toxin formation may be studied by culturing the organisms in a suitable liquid medium and injecting the bacteria-free filtrate into a susceptible animal (white mouse) which will develop the signs and symptoms of tetanus. It is always necessary to use two animals, one with the toxin alone and the other which has been given a prophylactic dose of antitoxin.

In the laboratory diagnosis of tetanus, it should be kept in mind that other non-pathogenic spore-bearing anaerobic bacteria are sometimes present in exposed wounds as saprophytes and their pre[illegible] may cause some confusion. Some of these organisms are *Cl. putrific*[illegible] *tetanomorphum, Cl. cochlearium, Cl. tertium, Cl. sphenoides* and a ho[illegible]ers for which special books should be consulted. Apart from minor [illegible]ces in the characters of growth and biochemical reactions, they never [illegible] any toxic effects on laboratory animals and are thus safely excluded.

CLOSTRIDIUM BOTULINUM

(Bacillus botulinus)

This is the casuative organism of botulism, a variety of food poisoning, due to eating tinned meat or imperfectly cooked meat which had been previously infected with the bacilli. The organisms were first isolated in 1896 by *van Ermengem* from infected pickled ham which was eaten by several persons in a Belgian music club. The name botulism was given to the disease because it originated from the eating of sausage (from *botulinus*, a sausage).

HABITAT:—The organism is widely distributed in the soil and may sometimes be found in the intestinal tracts of certain domestic animals, the chief of which is the hog. From such sources, the organisms may be disseminated by insects and the spores may be deposited on fruits, vegetables and other articles of diet. The organism does not multiply in the healthy living tissues but occurs as a harmless saprophyte.

MORPHOLOGY:—It is a fairly large bacillus, 4 to 6 μ long and about 1 μ thick, arranged either singly or in short chains. It forms an oval terminal spore which is thicker than the vegetative bacillus. It possesses 4 to 8 peritrichate flagella and is slightly motile. It has no capsule.

STAINING REACTION:—It is Gram-positive.

CULTURAL CHARACTERISTICS:—The organism is very strictly anaerobic. It grows well at low temperatures, near 20°C., but less luxuriantly at a temperature of 35°C. and over.

Glucose agar—On *surface glucose agar,* large translucent filamentous colonies are produced with a granular surface and fimbriated edge. Consistency of the growth is somewhat viscid. In *deep stab culture* in 1 per cent. glucose agar, there is a thin white column of growth and the medium is cracked and split, due to abundant formation of gas.

Nutrient broth with or without glucose:—There is a vigorous growth with general turbidity and a granular deposit at the bottom and copious evolution of gas. A rancid odour is produced owing to the formation of butyric acid.

BIOCHEMICAL REACTIONS:—This organism is **both proteolytic and saccharolytic,** the former property predominating.

Cooked meat medium:—Vigorous growth takes place with blackening of the meat. There is a rancid odour and much evolution of gas.

Litmus milk—Casein is precipitated and later digested with alkaline reaction.

Gelatin is readily liquefied.

It produces plenty of gas in all media, specially those containing glucose, the chief gaseous elements being hydrogen and methane.

VIABILITY:—The vegetative organism is no more resistant to heat, desiccation, sunlight and antiseptics than any other pathogenic bacterium, but its spores are extremely hardy. The latter can be destroyed by dry heat at a temperature of 180°C. for 5 to 15 minutes. It requires boiling for some hours before the spores are killed and they may remain alive in the autoclave for nearly 5 minutes. Thus, the enormous resistance of the spores to heat enables them to withstand tinning and cooking processes, specially if these are done inadequately.

TOXIN PRODUCTION:—Cl. botulinum produces, under strictly anaerobic conditions, a very powerful, soluble, extra-cellular toxin, which, on being absorbed through the alimentary tracts, produces the symptoms of botulism. This toxin is destroyed at a temperature of 80°C. but it resists the action of acids. It is not altered by the gastric juice in the stomach from where it is rapidly absorbed. Thus, the toxin of Cl. botulinum differs from those of C. diphtheriæ and Cl. tetani, (i) in being fairly stable and resistant, (ii) in producing its injurious effects when taken by the mouth and (iii) in not producing any toxin in the tissues of man and animals. In this respect, it differs very widely from the latter two exotoxin-producing organisms. Neither the injection of the bacilli into the tissues or circulation nor feeding by mouth is capable of producing symptoms. The potency of the toxin is very great and almost incredible. A dose of 1/1,000th of a c.c. is enough to kill a monkey and 1/20,000th of a c.c. will kill a rabbit or a guinea-pig in a day or two. All laboratory animals, including mice and chickens, are highly susceptible, whereas the cold-blooded animals, such as frogs and fish, are unaffected.

Types: Two main types (A & B), identical in morphology but distinguished by their toxin production, have been described. Antitoxin of one fails to neutralise the other. Type A is also distinguished from type B by the fact that the former is pathogenic for fowls and ferments salicin. Strains differing from types A and B have also been isolated and the name parabotulinus has been applied to them. These are generally non-proteolytic and do not ferment glycerol (cf. types A & B which ferment glycerol).

SEROLOGICAL REACTIONS:—When the toxin of Cl. botulinum is injected into a susceptible animal in repeated sublethal doses or when a man or an animal recovers from an attack of botulism, its homologous antitoxin is developed in the serum of that particular animal or man. This antitoxin, like that of C. diphtheriæ or Cl. tetani, completely neutralises the toxin. For commercial purposes, antitoxic serum is prepared by the immunisation of goats. When the bacilli, absolutely freed from the toxin, are injected intraperitoneally or intravenously into a rabbit, they give rise to the production of agglutinins, precipitins and complement fixing bodies in the blood of the injected animal. Such agglutinating serum is used for the diagnosis of botulism.

ANIMAL INOCULATION—All the laboratory animals are susceptible to the toxins by subcutaneous inoculation or feeding. The symptoms resemble those of acute bulbar paralysis.

PATHOGENICITY

When the toxin of Cl. botulinum is ingested, signs and symptoms of botulism develop. This may occur both in man and animals. It should be noted that the organism produces injurious effects entirely by its toxin.

Animals:—Of the animals, chickens, horses and cattle have been reported to have suffered from the intoxication due to feeding on infected beans, forage, corns and other materials which are stored up for fodder. All these animals suffer from varying degrees of weakness and muscular paralysis which, in severe cases, cause death.

Man:—In man, symptoms begin usually 24 hours after the consumption of the contaminated food. Unlike other varieties of food poisoning, there may not be any symptoms pointing to the gastro-intestinal canal. There are general weakness, lassitude, constipation, difficulty in swallowing and mastication, dry tongue with intense thirst and thickness of speech. Disturbances of vision are early symptoms and consist of diplopia, dilated pupils and photophobia, with paralysis of the muscles of the eye-lids. Thus, the symptoms resemble more or less those of belladonna poisoning. In very severe cases, paralysis of muscles becomes extreme and the patient dies of respiratory complications. There is no disturbance of sensation and general consciousness remains intact till near the end.

Morbid anatomy:—After death, all the organs are found congested and there may be extravasations of blood and thrombosis of vessels. No definite histological changes could be found in the central or peripheral nervous system. It has been suggested from the character of the symptoms which develop, that the main effect of the toxin falls upon the parasympathetic portion of the autonomic nervous system. (Cf. the action of Belladonna alkaloids).

It should be remembered that death is due to the effects of the absorbed preformed toxin and the bacilli of botulism never enter the general circulation nor are they injurious while inside the body. The poison is formed when the organism grows in food under anaerobic condition.

Difference between botulism and salmonella food poisoning:—The subject of botulism is intimately connected with that of bacterial food poisoning of which it forms only a sub-group. Whereas the variety caused by the Salmonella group of bacteria is an instance of an acute infection with growth and multiplication of the causative bacilli inside the alimentary tracts of the affected individual, botulism is entirely different, this being a typical example of intoxication. The toxin, in the latter case, exists already in a preformed condition in the contaminated food material where it remains in a very concentrated form and its subsequent consumption leads to its absorption through the mucous membrane of the alimentary tracts producing the toxæmia. Another very important difference between botulism and the salmonella food poisoning is the lack of spread of the disease in the former from person to person, as happens in the latter, due to the transference of the contaminating organisms through vehicles of food, drinks or fomites. Hence, secondary cases of human infection do not occur in botulism although domestic animals which feed on the remnants of the infected food may suffer from it. Botulism usually occurs as a sporadic outbreak due to eating tinned meat, fish or fruits or preparations of imperfectly cooked meat which has been kept for a long time, such as sausages, pickled ham, smoked meat, prepared foods, etc. In the case of an outbreak of botulism, the infected article of diet has been found to be consumed before final cooking or after inadequate cooking. The result has been that the toxin remains quite active and without much alteration. In some cases, the particular element of food may show some change, either in odour, colour or consistency, but in other cases, no noticeable alteration may be found. In the case of tinned food, gas may be found inside and there may be some rancid odour in the article. Owing to the incidence of the disease depending on such food materials, botulism is a very uncommon malady in our country where food is always cooked before consumption and where the use of tinned food stuffs has not yet found favour with the people in general.

Table showing the difference between the two types of bacterial food poisoning.

	Botulinum food poisoning.		*Salmonella food poisoning.*
1.	Causative organisms are anaerobic, spore-bearing and Gram-positive.		Causative organisms are aerobic, non-sporing and Gram-negative.
2.	An example of intoxication.		An instance of infection.
3.	Meat becomes infected after the animal has been slaughtered.		Meat was already infected at the time of slaughter, i.e., the animal was already sick.
4.	Bacteria produce the toxin outside the animal body and they cause no harm inside the body.		Bacteria enter the alimentary canal through contaminated food stuff and multiply inside, producing the symptoms.
5.	Secondary cases do not occur, except in animals which may feed on the remnants of the affected food.		Secondary cases occur due to person-to-person infection.
6.	Gastro-intestinal manifestations are absent.		Marked gastro-intestinal symptoms are present.
7.	Symptoms of Belladonna poisoning or paralysis of nerve-endings.		Symptoms resemble cholera or dysentery.
8.	Course is afebrile.	8.	Course is febrile.
9.	Duration is long.	9.	Duration is short.
10.	Onset is usually gradual.	10.	Onset is sudden.
11.	Treatment—Antitoxin.	11.	Treatment—Systemic.
12.	Mortality—60 to 70%.	12.	Mortality—1 to 2%.

THERAPEUTIC CONSIDERATIONS

Although a powerful antitoxic serum has been produced against the toxins of Cl. botulinum, it has not been found to be constantly efficacious in the treatment of actual human cases. In practice, the serum should be injected in fairly big doses, 50 c.c., preferably intravenously, and repeated every day till the patient recovers. It is necessary that the serum treatment be started as early as possible to neutralise the absorbed toxin. It is now known that antiserum of one type will not produce any beneficial effect on the other type. Hence, a polyvalent serum should be used.

Apart from the antitoxic serum, medicinal treatment should also be tried. Iodine and potassium permanganate may be [illegible] by the mouth as they both destroy the toxin. Alcohol has been k[illegible] to precipitate the toxin and hence, repeated small doses of brandy [illegible]icated. Olive oil retards the absorption of the toxin and liquid soap is said to neutralise it. Nervine stimulants, such as Nux vomica, are beneficial.

Prophylaxis:—Fresh food stuffs which are well cooked and eaten without much delay, are free from dangers of botulism. The main source is the tinned food and every care should be exercised to avoid it. In this respect, the Indian custom of cooking and eating has much to commend itself. If it is necessary to use tinned food, some points should be noted before using it. Bulging of the tin, the presence of gas bubbles, any alteration in the appearance or consistency of the food or the presence of a rancid smell, should at once put the consumer on guard and such a food should be totally discarded. The consumption of sausages and raw, pickled meat is always potentially risky and it is a very good thing that such a practice is not in vogue in our country.

LABORATORY DIAGNOSIS

This should comprise, mainly, the demonstration of the pathogenic effects of the toxin on laboratory animals and the isolation of the causative organisms from the suspected food material or the alimentary tracts of the affected person.

Demonstration of the effects of the toxin:—This may be done in one of the following ways:—

(*i*) A portion of the suspected food stuff may be given to a susceptible animal which on feeding on it, may show signs and symptoms of botulism.

(*ii*) The food may be suspended in saline or minced or ground up and then mixed with saline. This may be filtered and centrifuged and the supernatant fluid collected; or the fluid may be filtered through a porcelain filter to make it bacteria free. One to two c.c. of such a fluid may be injected into the peritoneal cavity of a mouse or a guinea-pig and the animal is watched for the development of the characteristic symptoms. Protection experiments may also be carried out, either by mixing the fluid with the corresponding antiserum or by giving an injection of antitoxin to the animal previous to its inoculation with the saline filtrate.

Isolation:—It requires a good deal of care and a long time, viz., about 10 days. This may be done by inoculating a suitable medium, preferably a fluid medium, with a little of the suspected material and incubating it under anaerobic conditions. This may then be heated to 80°C. for half an hour to destroy all the contaminating vegetative organisms, leaving only the resistant spores alive. After this process, a little of the heated culture may be planted either in deep glucose agar tubes (stab cultures) or on the surface of a plate and incubated anaerobically for about a week when the characteristic growth is obtained. From this culture, morphology,. biochemical properties, toxin production and animal experiments may be done to establish the identity of the bacteria.

When a patient has recovered from an attack, there is sufficient amount of antitoxin in his blood to be actually demonstrated. Such blood serum will be able to protect a susceptible animal from the effects of the toxin of Cl. botulinum.

GAS GANGRENE

Gas gangrene is a massive necrosis of tissues with gas formation and associated with a profound toxæmia. It is the result of inoculation of lacerated wounds with anaerobic bacteria of the clostridium group. The condition should be distinguished from the usual types of moist gangrene in which the evolution of gas is due to the putrefactive changes taking place in the tissues which are already dead or dying from some other cause. In gas gangrene, infection by anaerobic bacteria is the primary factor which leads to the death of tissues and gas formation, whereas in the other form, the bacterial infection is only a secondary phenomenon.

During the pre-Lister days, this variety of gangrene was very frequent and was the greatest obstacle to successful surgery. When antiseptic surgery came in to occupy the forefront, the incidence of the disease declined. But it flared up during the Great European War when it assumed a menacing proportion and attracted the keen attention of all the scientific men of the world. During this period, considerable progress had also been made regarding its etiology, pathology, treatment and prophylaxis. Now-a-days, we get instances of gas gangrene only in cases of severely lacerated wounds which are grossly soiled with dirt and materials contaminated with animal and human fæcal matter. Occasionally, this condition is met with as a complication in acute suppurative inflammation inside the peritoneal cavity, e.g., in acute appendicitis, intra-peritoneal abscess and gangrene of the gut.

Clinical picture:—The incubation period varies, but usually the symptoms begin to appear 12 to 24 hours after the injury. The onset is very sudden. A patient, left in a very good condition in the evening, may be found acutely ill and almost dying the next morning. Early symptoms are pain and numbness, malaise and restlessness. Vomiting is very common and in some cases, very troublesome. Signs of a severe toxæmia soon make their appearance. The patient looks very ill, with intense thirst, dry tongue, pallor, sunken eyes and extreme depression. The pulse becomes feeble, rapid and running, and the temperature subnormal. There is cold perspiration and extremities also become cold and cyanosed. The mental condition remains clear till the end. Death usually takes place suddenly from circulatory failure.

Pathological lesions:—The characteristic lesions consist of œdema, gas formation (Fig. 217) and necrosis of muscles and other tissues, the former being more involved than the cellular subcutaneous tissues. Hence, this gangrene is always associated with crepitus whi[illegible] may extend even to a very distant place, due to the gas travelling alon[illegible] planes of the muscles. There is also loss of function. The gas b[illegible] usually collect in between the muscle fibres which soon lose their [illegible] striations and which pass through the various stages of degen[illegible] processes until a complete coagulative necrosis sets in them. *Pari p*[illegible] affected muscles

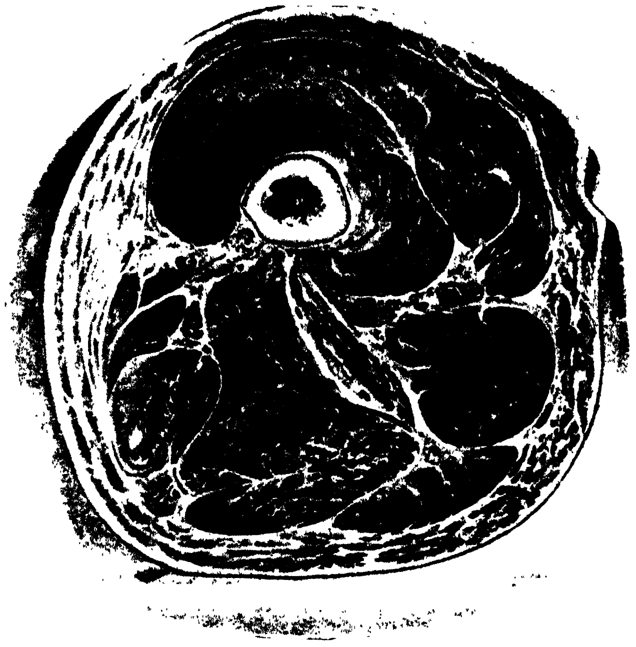


Fig. 217.

A transverse section of a right thigh showing the emphysematous condition of the muscles on the posterior aspect of the femur in a case of gas gangrene. There are bubbles of gas in the neighbouring subcutaneous fat.

(*From the British Journal of Surgery; by kind permission of John Wright and Sons, Ltd.*)

show characteristic colour changes (Figs. 218, 219, 220, 221). Towards the beginning, there is hardly any noticeable difference from the normal colour, but as the degenerative changes proceed, the colour becomes a dirty red, changing rapidly to a yellow and finally to a black colour, due to the chemical action of the sulphuretted hydrogen on the iron liberated from the broken down muscle-hæmoglobin. This takes place after the proteolytic digestion is established in the muscles, rendering the latter soft and diffluent.

The development of gas bubbles is astoundingly rapid. Towards the beginning, this gas mainly consists of carbon dioxide and hydrogen and is odourless, but with the progress of the infection, the smell soon becomes very fœtid, owing to the liberation of sulphuretted hydrogen and volatile ammoniacal bodies by the proteolytic group of bacteria.

In addition to the above changes, the blood vessels are damaged and thrombosis occurs and this further helps the necrotic process.

The toxins of the gas-gangrene-producing organisms, particularly those of the Cl. welchii, are powerful hæmolytic agents and when these are absorbed, they lead to considerable hæmolysis, resulting in hæmoglobinæmia and jaundice.

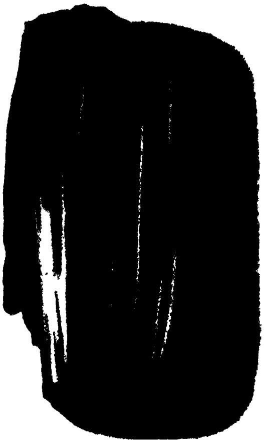


Fig. 218.

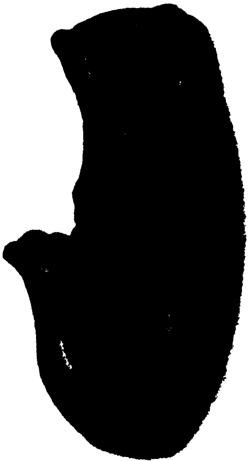


Fig. 219.

Fig. 220.

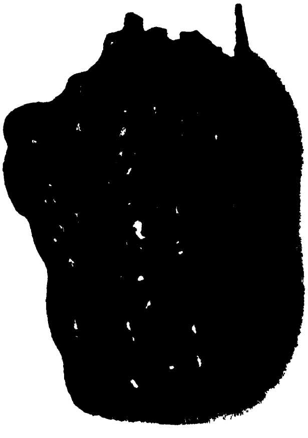


Fig. 221.

Fig. 218. A portion of the gluteus maximus muscle from a patient who died of acute peritonitis. The normal colour, sharpness of outline and general appearance of the fasciculi show that the muscle was healthy. **Fig. 219.** Early colour changes in a piece of muscle from the gluteus maximus in a case of gas gangrene. Note the brick-red colour of the muscle and the clear spaces once occupied by gas bubbles. The muscle at this stage is crepitant to touch, friable and non-contractile. **Fig. 220.** A further stage in the gangrenous process, showing the change of colour from brick-red to an olive green. **Fig. 221.** The end-stage of a muscle in gas gangrene, showing the greenish-black colour of the muscle and the glistening appearance of the surface. Such a piece of muscle is so soft that it tends to flatten out and spread over the surface on which it is placed.

(*From Keen's Surgery; after Sir Cuthbert Wallace; by kind permission of Saunders Company.*)

Although in milder cases, the organisms may remain localised, in all severe infections and specially before death, the m[illegible]thogenic members invade the system by passing into the general blo[illegible]am. Under such circumstances, the bacilli lodge in the liver, peritone[illegible]dneys, spleen and other internal organs which may show degeneration and gas formation. One very important noteworthy feature of this infection is the total absence of all inflammatory cellular reactions in the lesions at all stages and from the beginning to the end. It is for this reason that there is no pus formation but only a sero-sanious discharge from the lesions and a smear examination of such an exudate shows very few leucocytes. *Histological examination* reveals no leucocytic emigration but only coagulative necrosis of the tissue elements (Figs. 222, 223, 224, 225, 226).

The following conditions act as **predisposing factors** *in the production of gas gangrene:—*

1. Extensive laceration and crushing of tissues, which often occur in shell and bullet wounds, serious street accidents, compound fractures with severe lacerations, specially when these are complicated by large extravasations of blood.

2. Circulatory disturbances caused by division or occlusion of the main artery or the prolonged pressure of a tourniquet or tight bandage applied as a first aid to stop bleeding.

3. Imperfect drainage and consequent retention of the extravasated blood and other discharges brought about by tight plugging or dry hard dressing.

4. Buried missiles, foreign bodies, portions of dirty clothings, pieces of bone from comminuted fracture, etc.

5. Hæmorrhage, shock, exposure to cold and in fact, lowering of vitality due to any cause.

Bacteriology of gas gangrene

This subject was exhaustively studied during the last European War and as a result, it was found that a large number of bacteria was usually more common in gas gangrene than a single micro-organism. Besides the spore-bearing anaerobes, aerobic cocci and bacilli also lodge in the wound. Just to summarise, in gas gangrene, the bacteria may be broadly divided into the following groups.

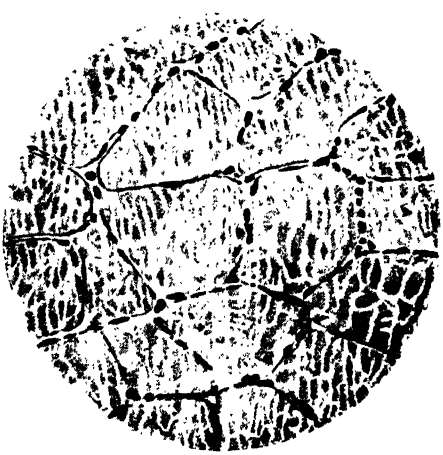


Fig. 222.

Transverse section of a normal muscle.

1. *Most virulent, acutely pathogenic organisms* which comprise **Cl. welchii, Cl. septique** and **Cl. œdematiens.** All of them are strongly saccharolytic.

2. *Organisms of less pathogenicity and doubtful toxicity,* comprising **Cl. fallax** and **Cl. histolyticum.**

3. *Organisms which by themselves have no pathogenic action,* but

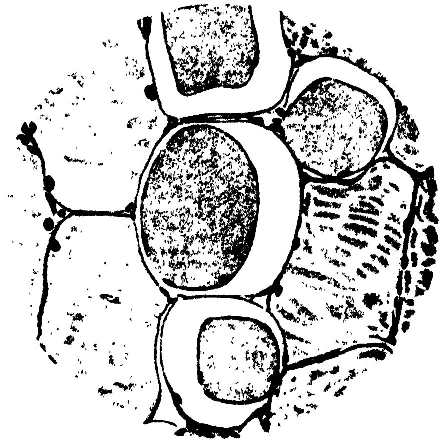


Fig. 223.

Transverse section of a muscle from the advancing edge of gas gangrene. Note the marked contrast between the dead coagulated fibres which are separated off from their sheaths, and the paler normal fibres.

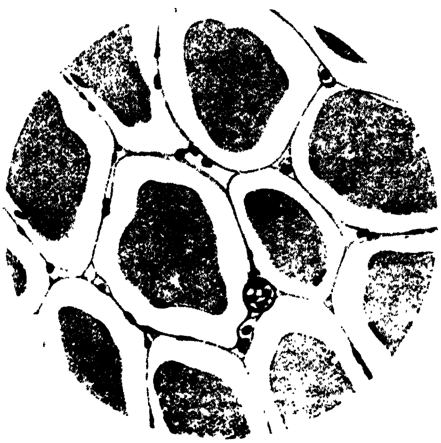


Fig. 224.

All the fibres are here degenerated, but their sarcolemmal nuclei still appear intact Separation of the fibres from their sheaths is everywhere complete.

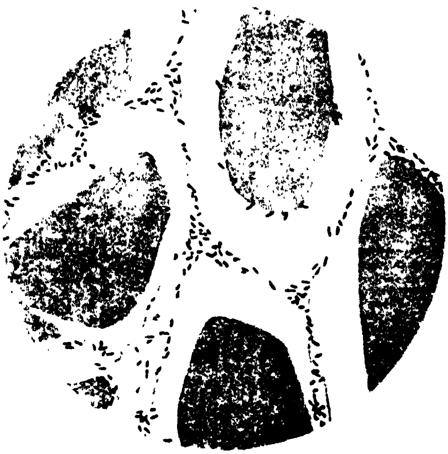


Fig. 225.

This shows the distribution of the organisms at a comparatively early stage. There are large numbers of bacilli in the reticulum, but they have not yet invaded the fibres. This stage is slightly more advanced than that depicted in Fig. 224, and the nuclei of the sarcolemma have disappeared.

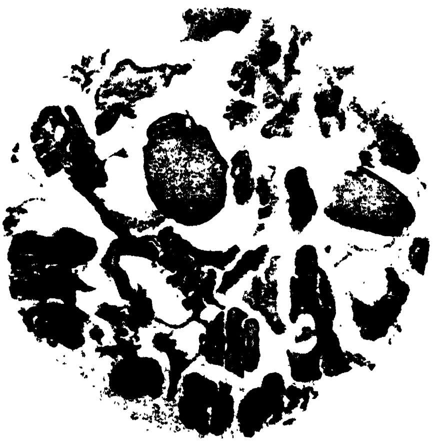


Fig. 226.

A late stage of gas gangrene, showing complete disintegration of muscular fibres and reticulum. In this material organisms were present in large numbers, and frequently in the substance of the fibres.

(*From Keen's Surgery; after Sir Cuthbert Wallace, by kind permission of Saunders Company*).

nevertheless, exert a very harmful effect in the pr[illegible] of each other and also of the more important pathogens. Most of [illegible] have little action on carbohydrates but they are highly proteolytic. [illegible] typical organism of this group is the **Cl. sporogenes.**

4. **Aerobic bacteria,** such as *streptococci, staphylococci* and *pyogenic bacilli,* may also be found, specially towards the beginning. The rôle played by these cocci and bacilli in a lacerated wound is always favourable to the germination of spores of the anaerobic bacteria and their subsequent multiplication. By using up a large amount of oxygen for themselves, they lower the oxygen tension in the wound and help to produce an anaerobic condition which is absolutely essential for the growth of the pathogenic anaerobes.

The presence of the *spores of Cl. tetani* in a grossly contaminated, lacerated wound should always be remembered. Although this organism is not responsible for the production of gas gangrene, its presence in such wounds is no less risky, specially when we have seen that the effects of the toxins (aggressins) of Cl. welchii and Cl. septique are always favourable to the growth of Cl. tetani.

Pathogenesis:—We have already seen that the gas-gangrene-producing organisms are all saprophytes, inhabiting the intestines of men and animals, and flourishing on decaying organic matter, either animal or vegetable in nature; that all are spore-bearing organisms and, owing to the high degree of resistance of the spores, they are able to survive for long periods in the soil and dust. Decomposing blood clots and dead or partially devitalised tissues form the best medium for the germination of spores and the growth and multiplication of these saprophytic organisms. We have also seen that these bacteria owe their action mainly to the extra-cellular toxins which not only give rise to a serious constitutional poisoning but also produce a very marked negative chemiotaxis through their powerful *aggressins,* thus preventing any resistance which may be set up by the cellular defences of the body. When a severely lacerated wound becomes infected with the gas-gangrene-producing organisms, two things may happen.

If there be not much dead and necrotic material and if sufficient tissue vitality is preserved, large numbers of phagocytes come to the area of the injury and destroy the spores as well as the bacilli which may have germinated, before the latter can have time to produce their toxins in sufficient concentration. For, as we have seen already, it is this toxin which is the most important element in repelling the leucocytes and helping the bacilli to multiply and establish themselves firmly. It may be said that "the disease really begins, not when a wound has become infected with the pathogenic anaerobes, but from the moment when a group of these bacteria has been enabled to surround themselves with a toxin, sufficiently concentrated to abolish the local defences of the tissues." Once this has occurred, the organisms multiply, produce more toxins and rapidly invade the tissues.

When sufficient dead and necrotic organic material is present in the wound and the vitality of the tissues is also lowered very much, the ideal conditions for this infection are available. The spores germinate and the bacilli rapidly multiply and produce toxins. Towards the beginning, the saccharolytic group is the first to develop. These rapidly convert the muscle carbo-hydrates into carbon dioxide, hydrogen and organic acids. The last named product, when formed in sufficient concentration, would tend to inhibit the growth and activity of the saccharolytic group, but in the actual process of infection, this cannot take place, because of the subsequent development and the biochemical activities of the proteolytic group. These latter organisms attack the proteins and break them up, liberating sulphuretted hydrogen and volatile ammoniacal bodies which act as neutralising agents against the organic acids. The presence of alkaline mineral salts of calcium, magnesium and potassium in the soil and the exuded materials from the tissues which are alkaline in reaction, also help to neutralise these acid products of carbohydrate fermentation. After the neutralisation, there is luxuriant growth of the bacilli with formation of toxin and the latter, after absorption, paralyses the suprarenal glands and causes death by toxæmia and collapse.

To sum up:—

1. Infection of a lacerated wound with (a) pyogenic bacteria and (b) spores of anaerobes.
2. Production of an anaerobic condition by various factors.
3. Spores of the anaerobes germinate and the bacilli multiply, producing toxins (aggressins) which repel the leucocytes, thereby abolishing the local defences of the host.
4. Saccharolytic group of organisms (Cl. welchii) acts on muscle carbohydrate, producing H, CO_2 and organic acids. The latter inhibit further multiplication of Cl. welchii.
5. Proteolytic group of organisms (Cl. sporogenes) now acts on muscle protein, producing H_2S and volatile ammoniacal bodies. The latter neutralise the acids and thus enhance the growth of the Cl. welchii.
6. As a result of this symbiosis between the two groups of organisms, Cl. welchii, one of the most important pathogenic members, grows luxuriantly, liberating a large amount of toxins. This leads to an intense toxæmia (sapræmia) which paralyses the suprarenals and causes death.

Therapeutic considerations

The introduction of the antiserum against the organisms of gas-gangrene has reduced both the incidence and the mortality of the disease. To be of any service, it is necessary that the injections are given in big doses (20,000 units), preferably intravenously and as early as possible. It is also necessary to use a polyvalent serum as the infection is usually a mixed one. The injections should be repeated frequently (every 8 to 12 hours). In cases of severe infection with advanced toxæmia, prompt intravenous injection of a large dose of serum forms the only chance of a cu[illegible]

In civil practice, in cases of suppurative ap[illegible]tis and intestinal obstruction, where anaerobic toxæmia has been fo[illegible] play an important part, large doses of antiserum should be admi[illegible] at the earliest opportunity.

Prophylaxis:—In all lacerated wounds, suspected to be grossly contaminated, injection of a polyvalent " antigas " serum should be given as a prophylaxis. It is always desirable to combine the antigas-gangrene with antitetanic, serum, as both the infections may co-exist. Similarly, in major surgical operations for intra-abdominal inflammatory and other conditions, a prophylactic dose (4,000 units) of antigas serum should be given before the operation is undertaken.

Surgical prophylaxis:—

(1) As deficient blood supply favours anaerobic infection, special care should be taken to avoid the prolonged application of a tourniquet, tight bandage and similar appliances.

(2) Dry hard dressings and tight plugging should not be used in infected and lacerated wounds. These impede circulation and prevent efficient drainage. Pent-up secretions aid anaerobic infection.

(3) Scrupulous cleaning of wounds and if possible, complete excision of a grossly soiled lacerated wound should be undertaken at the earliest moment.

(4) As shock and hæmorrhage lower general vitality and favour tissue devitalisation, all attempts should be made to combat these complications.

In dealing with a case of gas gangrene, certain precautions should be taken. These are as follows:—

(a) Isolation of the case is absolutely essential to prevent the spread of contamination to other cases, when it is in a hospital.

(b) All dressings soaked with the discharge from the wound should be burnt.

(c) Gloves should be used in handling such a case and all instruments should be set apart for the case. These should either be discarded or properly sterilised, preferably in an autoclave.

Laboratory diagnosis

Fluid from an infected wound or a portion of the infected muscle may be selected for examination. *Microscopical examination* of a smear made from such a material and stained by Gram's method may help to determine the nature of the organisms present. The material is then *inoculated into a suitable medium, preferably cooked-meat medium.* This will of course allow the growth of all kinds of organisms, including the anaerobes. A little of this mixed growth is heated to 80°C. for 20 minutes, in order to destroy all aerobic and vegetative anaerobic forms and then plated on blood or glucose agar and incubated anaerobically. Single colonies may form which may then be studied in the usual way. *Agglutination tests* and *animal inoculation experiments* should always be done for identification and study of toxin-formation respectively.

The identification and separation of the individual bacteria require expert knowledge and much technical skill. The difficulty is specially increased because different varieties of the anaerobes are always intimately mixed up.

For example, Cl. sporogenes and Cl. welchii may be so mixed up that they are separated with difficulty after prolonged and repeated cultivation in glucose broth.

A very useful method of *isolating the organism in pure form* is to emulsify the exudate from a suspected wound and to inject it intravenously into a rabbit. The animal is killed after a few minutes and then placed in an incubator for 5 to 6 hours. The animal will be found to be distended with gas. The organisms may then be isolated from the liver or heart's blood almost in pure form.

In the final *identification*, protection experiments should always be done For instance, if a mixture of Cl. welchii, Cl. septique and Cl. œdematiens be inoculated into a guinea-pig which has already received a prophylactic injection of anti-serum prepared against the latter two bacteria, then Cl. welchii alone will grow and infect the general circulation. Thus, by suitable combinations, the different pathogenic varieties may be identified.

Isolation of Cl. welchii from stool may be done in the following way:—Five c.c. of a fæcal suspension in saline are inoculated into a tube of freshly boiled milk. The tube is heated to 80°C. for half to one hour to destroy all the vegetative forms of bacteria. After this, it is incubated anaerobically. The " stormy fermentation " indicates the presence of Cl. welchii. Plating may be done subsequently for purification.

ORGANISMS OF GAS GANGRENE

SACCHAROLYTIC GROUP—The important members of this group which causes gas gangrene are Cl. welchii, Cl. œdematiens, Cl. septique, and Cl. fallax.

CLOSTRIDIUM WELCHII

(*B. welchii*).

Synonyms:—*B. aerogenes capsulatus, B. perfringens, B. enteritidis sporogenes.*

First described by **Welch** and **Nuttall** in 1892.

Morphology:—It is a long, rod shaped bacillus, forming a large, oval and central or subterminal spore. It possesses a very well-marked capsule inside the animal body. It has no flagella and is non-motile.

Cultural characters:—It grows in all the usual laboratory media but requires strict anaerobic conditions and prolonged incubation, even as long as 15 days.

On the *surface of a glucose agar plate*, the colonies are discrete, round, opaque and the edges slightly crenated. Their consistency is butyrous.

In *deep stab cultures* and *shake cultures*, there is so much gas formation that the entire medium is broken up and scattered.

There is good growth *in broth*, with the produc[illegible] gas and a sour smell.

Biochemical reactions:—The organism is mainly saccharolytic with little or no proteolytic action. It is commonly termed " gas bacillus," because of its production of much gas.

Cooked meat medium—Luxuriant growth takes place in this medium, producing acid and gas and a sour odour, the meat being turned pink in colour.

Litmus milk—It produces acid, gas and clotting. The formation of gas is so abundant that its action on this medium is referred to as "stormy fermentation" (Fig. 227).

Sugar fermentation—It ferments glucose, maltose, lactose and saccharose with the formation of acid and gas. During the process of growth, specially in a medium containing sufficient fermentable carbohydrates, there is production of a large amount of organic acids which inhibit the growth of the bacilli and the latter may ultimately die out. This is a very important factor and has already been discussed in connection with the pathogenesis of gas gangrene.

Gelatin is liquefied.

Coagulated serum medium or egg medium—There is no digestion or liquefaction of proteins in these media.

Blood agar—The organism produces marked hæmolysis.

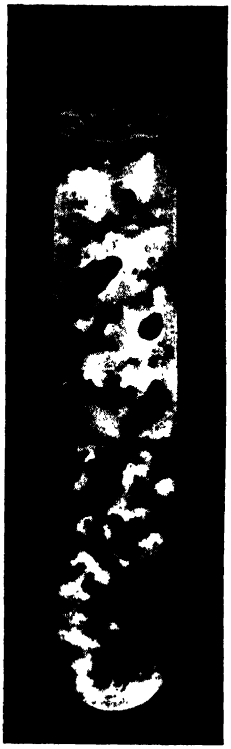


Fig. 227.
Growth of *Clostridium welchii* in litmus milk, showing stormy fermentation.

Viability:—The vegetative organisms cannot stand the action of heat, sunlight and desiccation more than any other pathogenic micro-organism. The spores, on the other hand, are very resistant and may remain alive for months and years. It requires boiling for about half an hour to destroy these spores completely.

Toxin production:—The organism produces a very powerful exotoxin which causes tissue necrosis and damage to the supra-renals. Like other pathogenic anaerobes, it produces aggressins which produce a negative chemiotaxis and thereby repel the leucocytes from the area of infection. Some strains produce hæmolysin and leucocidin.

Serological considerations:—Injection of washed spores or bacilli into the peritoneal cavity or blood of laboratory animals gives rise to the production of specific agglutinins. By repeated injections of the toxin in sublethal doses in horses, a powerful antitoxic serum has been produced. The latter neutralises the extracellular toxin *in vitro* as well as *in vivo*. Hence, when animals are previously injected with a sufficient dose, it protects them when subsequently inoculated with a broth culture of the organism.

Pathogenicity:—All the pathogenic effects are due to the action of its extracellular toxin. When a susceptible laboratory animal is inoculated with a little broth culture of the Cl. welchii, there is a local swelling, spreading œdema with gas formation and death in 24 to 48 hours. The organisms usually invade the internal organs and clumps of bacilli may be found inside the capillary vessels of the liver, spleen, kidneys and lymphatic glands. Histologically, the picture presents a very characteristic appearance. The muscles and other tissues undergo a sort of quiet necrosis without any accumulation of leucocytes—a characteristic unlike that which is always such a constant feature in all inflammations induced by pyogenic and other pathogenic micro-organisms. The animals which are particularly susceptible to Cl. welchii are pigeons, guinea-pigs and mice.

In man, Cl. welchii is one of the most important agents in the production of gas gangrene.

Rôle of the Cl. welchii in General Surgical Conditions:—Apart from the production of gas gangrene in infected wounds, these anaerobic organisms are gradually being found to be important pathogenic elements in various surgical conditions in civil life. For instance, gas gangrene producing anaerobes have been isolated from suppurative inflammatory conditions of the vermiform appendix, as well as after major abdominal operations. The toxins of these organisms add to the seriousness of the primary condition and their invasion into the muscles and cellular tissues of the abdominal wall produces necrosis and gas production. In cases of strangulation, intussusception, volvulus and similar conditions causing acute intestinal obstruction and gangrene of a portion of the gut, the Cl. welchii and its pathogenic associates (which are inhabitants of the intestinal tract) find favourable conditions for growth and multiplication. The result is a very serious grade of toxæmia with invasion of other tissues of the body. The very high degree of mortality in these cases has been found, of late, to be due, in great part, to the toxins of Cl. welchii.

CLOSTRIDIUM SEPTIQUE

Synonyms:—*Vibrion septique (Pasteur), B. œdematis maligni (Koch).*

Pasteur and **Joubert,** 1877.

Morphology—This is a thin, rod shaped bacillus which forms oval, subterminal endospores. It possesses many peritrichate flagella and is actively motile. In a fresh specimen of blood of a guinea-pig dying of this infection, the organisms appear in long undulating chains which move among the blood cells as serpents in the gras [illegible] eur). Such serpent-like forms are also seen in a wet preparation fro [illegible] liver of the guinea-pig. This is an important feature by means of wh [illegible] organism can be distinguished from Cl. chauvœi with which it clos [illegible] mbles.

Cultural characters:—It grows practically in all the common laboratory media in the same way as Cl. welchii, with much gas formation and complete breaking up of the medium. This is particularly so in the case of a deep stab or shake culture. There is also production of a rancid odour. The *surface colonies*, however, differ from those of Cl. welchii. They are more or less transparent, and somewhat spreading, with markedly fimbriated margins resembling a fern.

Biochemical reactions:—The organism is mainly saccharolytic with very little proteolytic action.

Cooked meat medium—In the cooked meat medium, there is acid and gas production with sour odour, the meat being turned pink in colour.

Litmus milk is clotted and turned acid. The process of clotting is usually delayed to 3 to 4 days and the formation of gas is not so pronounced as in the case of Cl. welchii.

Sugar—It ferments the common sugars with the production of acid and gas.

Gelatin is liquefied completely. There is neither digestion nor liquefaction of protein in the *coagulated serum or egg medium*.

Toxin production—The organism produces a very powerful soluble exotoxin and hæmolysin.

Serological reactions—The washed bacilli, when injected into rabbits, can give rise to the production of specific agglutinins. Such agglutinating sera are used for the identification of the organisms. By this means, four serologically different types of the organism have been isolated.

The injections of exotoxin into an animal give rise to the production of an antitoxin in the blood of that animal. Such antitoxin will neutralise the toxin and its effects, when injected in suitable amounts into an infected animal. Commercially, large amounts of anti-toxic sera are produced by immunising horses.

Pathogenicity—The pathogenic action of this organism depends entirely on its extra-cellular toxin. When a little broth culture is injected into the tissues of an animal, there is developed an extensive œdema and gas formation at and near the site of the inoculation and the animal dies of toxæmia within 12 to 24 hours. *Post mortem*, the muscle fibres which are deep red in colour are separated by œdema and gas formation and there is discharge of a dirty blood-stained fluid. The bacilli enter the blood stream at a very early stage and are found in large numbers in the liver, spleen and other internal organs.

Naturally, this organism causes fatal diseases in animals, such as black-leg and an acute hæmorrhagic inflammation of the stomach and duodenum of sheep.

In man, Cl. septique constitutes one of the most important elements in the causation of gas gangrene.

CLOSTRIDIUM ŒDEMATIENS

(B. novyi)

Synonym:—*Cl. novyi.*

Novy, 1894; **Weinberg** and **Séguin,** 1915.

This is another spore-bearing anaerobic bacillus which is an etiological agent of some importance, in the production of gas gangrene in man. It resembles the previous two organisms in morphology, staining reactions and cultural characteristics, and like them, produces a very powerful extra-cellular toxin which is fatal to laboratory animals. When a culture is injected into the animal tissue, there is production of gangrene with œdema and gelatinous exudation. The gas formation is, however, not very marked as in the other two organisms. *The surface colonies* are irregularly rounded and translucent with a sponge-like surface. The organism is *mainly saccharolytic* with very little proteolytic action.

CLOSTRIDIUM FALLAX

(B. fallax)

Weinberg and **Séguin,** 1918.

This organism resembles Cl. welchii and for this reason was mistaken for it, hence the name fallax. The organism is motile. The spores are oval and subterminal but are not readily formed. The organism is mainly saccharolytic and has no proteolytic action. It litmus milk it produces acid and clotting; sometimes gas is formed but this occurs slowly (cf. Cl. welchii). An exotoxin is formed and the organism is pathogenic to guinea-pigs when freshly isolated. Its relation to the etiology of gas gangrene has already been considered.

Other members of the saccharolytic group of anaerobes may often be found in gas gangrene associated with the more important pathogenic groups, but their individual pathogenicity is doubtful.

PROTEOLYTIC GROUP—The organisms [illegible]led in this group are Cl. histolyticum, Cl. sporogenes, Cl. ærofœtic[illegible] [illegible]d Cl. bifermentans, of which the first two are only important.

CLOSTRIDIUM SPOROGENES

(B. sporogenes)

Described by **Metchnikoff** in 1908.

The organism is motile and possesses an oval and subterminal spore. *Surface colonies* are wooly and rhizoid. It is both proteolytic and saccharolytic but the latter action is not so marked. It does not produce any soluble exotoxin and is non-pathogenic to laboratory animals. In the production of gas gangrene, it appears to exalt the virulence of Cl. welchii.

CLOSTRIDIUM HISTOLYTICUM

(B. histolyticum)

Weinberg and **Séguin**, 1916.

The organism resembles Cl. sporogenes but is actively proteolytic. It produces a soluble exotoxin and is pathogenic to laboratory animals. Intramuscular injection of 1 c.c. of a young broth culture into a guinea-pig causes at the end of 12 to 24 hours digestion of the skin and muscles down to the bone. This is a very striking feature, because, inspite of the intense local lesion, the health of the animal remains unaffected.

Rôle of the proteolytic group in gas gangrene.

The proteolytic group of the anaerobic bacteria, although present in gas gangrene, can never produce the lesions by themselves. They confer a very offensive and foul odour to their culture and to gas gangrene. They are not very pathogenic to laboratory animals nor do they produce any symptoms of general intoxication, like the saccharolytic group. Owing to their power of digesting and liquefying proteins, they produce a large amount of tissue-liquefaction which provides a good pabulum for the pathogenic saccharolytic group to grow and multiply. Another very important factor is the liberation of a very considerable amount of ammonia during protein decomposition and this helps to neutralise the large quantities of organic acids which are liberated by the saccharolytic group and which always tend to inhibit and limit the activity of the latter. Thus, it will be seen that the proteolytic anaerobic bacteria are always intimately associated with the pathogenic members of the gas gangrene producing bacteria and are present in the lesions in symbiosis.

For the use of the coloured diagrams and photomicrographs of gas gangrene our best thanks are due to the British Medical Research Committee, to their artist, and to the editor of the British Medical Journal.

Table showing the properties of the important members of the Clostridia group.

	Gram's stain.	Motility.	Spores.	Surface colonies.	Cooked meat medium.	Litmus milk.	Exotoxin.	Pathogenicity.	
								Guinea-pig.	Man.
Cl. welchii (S, p) ..	+	o	Oval, subterminal.	Round, opaque with wavy margins.	No digestion, turned pink.	AC stormy fermentation.	+	+	Gas Gangrene.
Cl. septique (S, p) ...	+	+	,,	Transparent and spreading.	,,	AC	+	+	,,
Cl. œdematiens (S, p)	+	+	,,	Irregularly round, translucent, with sponge-like surface.	,,	AC	+	+	,,
Cl. fallax (S) ...	+	+	,,	Round, granular, with crenated edge.	,,	AC	weak	+ when freshly isolated.	,, Occasionally.
Cl. tertium (S) ...	+	+	Oval, terminal.	Round, translucent, with wavy margins.	,,	AC	o	o	o
Cl. chauvœi (S, p) ...	+	+	Oval, subterminal.	Transparent fern-like edge.	,,	AC	+	+	o
Cl. histolyticum (P, s)	+	+	,,	Flat, with crenated edges.	Digestion with blackening.	Digestion.	weak	Local lesion.	Gas gangrene occasionally.
[illegible]*es* (P, s) ...	+	+	,,	Wooly, rhizoid.	,,	,,	o	o	Enhances the growth of saccharolytic group of gas gangrene organisms.
Cl. botulinum (P, s) ...	+	+	,,	Transparent filamentous, with fimbriated edges.	,,	,,	++	+	Food-poisoning.
Cl. tetani (p) ...	+	+	Spherical terminal.	Feathery, tendency to spread.	Evolution of gas without any digestion and blalcken-ing.	o	++	+	Tetanus.

P and S = marked proteolytic and saccharolytic action; p and s = slight proteolytic and saccharolytic action.
+ = positive; ++ = marked positive; o = nil or no change; AC = acid and clotting.

CHAPTER XXVII

COLI-TYPHOID-DYSENTERY GROUP OF ORGANISMS

(Gram-negative intestinal organisms belonging to the genus **Bacterium.***)*

This group is divided into 3 sub-groups as follows:—

I. **Escherichia** or *the colon group.*

1. *Escherichia coli* (B. coli communis).
2. *Escherichia communior* (B. coli communior).
3. *Escherichia acidi lactici* (B. acidi lactici).
4. *Aerobacter ærogenes* (B. lactis ærogenes).
5. *Aerobacter cloacæ* (B. cloacæ).
6. *Proteus vulgaris* (B. proteus).

These are found to be the normal inhabitants of the intestinal tracts of man and other animals. Ordinarily, these appear to be non-pathogenic; but, under abnormal conditions, they may cause acute or chronic infective lesions in the urinary tracts and elsewhere.

II. **Eberthella** or the *typhoid-dysentery group.*

1. *Eberthella typhi* (B. typhosus).
2. *Eberthella dysenteriæ, Shiga* (B. dysenteriæ, Shiga).
3. *Eberthella paradysenteriæ* (B. dysenteriæ, Flexner).
4. *Eberthella dysenteriæ, Schmitz* (Schmitz bacillus).
5. *Eberthella dysenteriæ, Sonne* (Sonne's bacillus).

III. **Salmonella** or *the food-poisoning group.*

1. *Salmonella paratyphi* (B. paratyphosus A).
2. *Salmonella schottmulleri* (B. paratyphosus B).
3. *Salmonella hirschfeldi* (B. paratyphosus C).
4. *Salmonella enteritidis* (B. enteritidis, Gærtner).
5. *Salmonella ærtrycke* (B. ærtrycke).
6. *Salmonella morgani* (B. morgan No. 1).
7. *Salmonella typhi murium* (B. typhi murium).
8. *Salmonella suipestifer* (B. suipestifer).
9. *Salmonella psittacosis* (B. psittacosis).
10. *Salmonella gallinarum* (B. gallinarum)—Non-motile.
11. *Salmonella pullorum* (B. pullorum)—Non-motile.

GENERAL CHARACTERS OF THE GROUP

Morphology:—These organisms vary in size, measuring about 2 to 4 μ in length. Most of them possess *multiple peritrichate flagella,* with the exception of the dysentery group of organisms. They are all non-sporing and non-capsulated.

Staining reactions:—They stain readily with all aniline dyes and are all Gram-negative. Special stain is necessary for exhibiting flagella.

Cultural characters:—They are easily grown in ordinary culture media. Special media are, however, used for their isolation. The general basis of these media depends on the presence of lactose, an indicator and an inhibiting agent for other bacteria. Incorporation of telluric acid and brilliant green in the medium assists the growth of pathogens and inhibits that of the non-pathogens.

Biochemical reactions:—

1. *Sugar fermentation*—Escherichia group ferments lactose, whereas Eberthella and Salmonella groups do not ferment this sugar. In glucose, Salmonella and Escherichia groups produce acid and gas, whereas Eberthella group produces acid only.

2. *Litmus milk*—Two types of reaction are found in this medium. The one is an immediate reaction which occurs during the first 24 hours and is characterised by the decomposition of the lactose in the milk with the liberation of acid which produces curdling, e.g., in Escherichia group. The other which is a remote reaction is characterised by the production of alkalinity due to the breaking up of the proteins, e.g., in Eberthella and Salmonella groups.

3. *Indol formation*—This is usually negative with the Eberthella and Salmonella, but positive with the Escherichia, groups.

4. *Gelatin*—None of the members liquefy gelatin, except Proteus vulgaris and Aerobactor cloacae.

Classification:—The sugar fermentation reactions form an important means of differentiating the various members of the group. The main basis of the classification rests on their power to ferment lactose or not, i.e., the first differentiation is made into lactose fermenters and non-lactose fermenters. The latter are further differentiated by their action on glucose as shown in the table below:—

Lactose:-

Lactose fermenters.
Escherichia group.
Exception—Proteus
vulgaris.

Non-lactose fermenters.
Eberthella & Salmonella groups.
|
Glucose.

Fermentation with the production of acid only
(Non-gas producer).
Eberthella group (Typhoid and Dysentery
bacilli).

..ation with the production
' and gas (Gas producers).
..lmonella group.

Character of colonies on the special media

	Lactose fermenters.	*Non-lactose fermenters.*
MacConkey's medium ...	Rose pink colonies.	Colourless colonies.
Conradi Drigalski medium	Red, opaque and large colonies.	Blue, transparent and small colonies.
Endo's medium ...	Red colonies.	Colourless colonies.
Brom-cresol-purple medium	Yellow colonies.	Purplish blue colonies.

Serological reaction:—Pathogenic members of the group produce specific and group agglutinins. This fact can be utilised in two ways, *firstly*, to identify the organism and *secondly*, to diagnose the disease.

Pathogenicity:—Eberthella and Salmonella groups are distinctly pathogenic. Escherichia group is non-pathogenic under normal circumstances.

EBERTHELLA TYPHI *(Bacterium typhosum)*

(Bacillus typhosus)

The causative organism of typhoid fever in man.

HISTORICAL:—The organism of typhoid fever was first described in the year 1880-81 by *Eberth* who observed it in the intestinal ulcer and in the spleen of autopsy cases of typhoid fever. *Gaffky*, in 1884, first isolated the organisms in pure culture from the spleen. He not only saw the bacteria referred to by Eberth but also investigated their cultural characters. The organism is commonly called B. typhosus and it is sometimes referred to as *Eberth-Gaffky bacillus*. Its new nomenclature is *Eberthella typhi* (Bacterium typhosum).

HABITAT:—The bacilli are not present normally in the intestinal tracts of man but only under pathological conditions. They are always found in the excreta of patients suffering from typhoid fever, as well as of carriers.

MORPHOLOGY:—The organism is a rod-shaped bacillus, measuring about 2 to 4 μ in length by 0·5 μ in breadth. It is very actively motile and possesses multiple *peritrichate flagella* (Fig. 228), the number of which varies from 8 to 14. The individual flagellum is much longer than the bacillary body, being 6 to 8 μ in length. Special stains are necessary to demonstrate the presence of flagella. It is a *non-sporing* and *non-capsulated* organism.

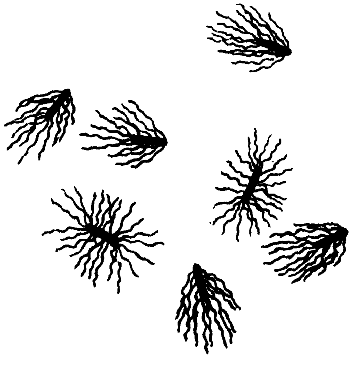


Fig. 228.
Eberthella typhi.
Note the peritrichate flagella.

STAINING REACTIONS:—With aniline dyes, it is stained readily and is Gram-negative.

CULTURAL CHARACTERS:—The bacilli grow quite easily in ordinary media. In *broth*, there is an uniform turbidity. There may be a pellicle formation on the surface, specially when it is grown for a few days. In *nutrient agar*, the organism forms small, clear, translucent colonies.

BIOCHEMICAL REACTIONS:—The organism ferments glucose, maltose and mannite, with the production of acid only but no gas. It has, however, *no action on lactose*. It does not liquefy gelatin and never forms indol. In litmus milk, the organism produces a faint trace of acidity without any coagulation of the medium (a control tube should always be used).

Character of the colonies in special media

MacConkey's medium—colourless.

Endo's medium—colourless.

Conradi-Drigalski's medium—blue.

Brom-cresol-purple medium—purplish blue.

Wilson-Blair's medium—black.

For the preparation of selective media, see page 68.

COLONY VARIATION:—During repeated subcultures in artificial media, two main types of colonies are observed (Fig. 229). The one, the *smooth type*, consists of normal bacilli, possessing all the typical qualities of E. typhi (B. typhosus), viz., motility, agglutinating property and antigenic power. Pathogenicity of such organisms is normal.

Fig. 229.
Smooth (S) and rough (R) colonies of *lla typhi.*

The other, the *rough type*, consists of bacil are self-agglutinating, possess no antigenic power and are avirulent.

Note:—They retain these acquired char s even after suitable subculture and animal passage.

VIABILITY:—E. typhi (B. typhosus) cannot exist for a long period outside the human body. Its existence depends, to a large extent, on the following conditions:—

(1) *Temperature*—The bacilli rapidly die out when exposed to a temperature of 60°C.

(2) *Sunlight*—When exposed to direct sunlight, the bacilli are readily killed.

(3) *Drying*—They cannot withstand desiccation.

(4) *Presence of Escherichia coli* or other soil bacteria definitely inhibits and ultimately destroys the growth of E. typhi.

Thus, the conditions met with outside the human host are not favourable to their prolonged existence. They can, however, survive long enough to be capable of transmitting the disease. For instance, they remain alive in a sample of stool from 2 days in the superficial portion to about 15 days in the centre of the fæcal mass. When they gain entrance into water, they may live for another 5 to 6 days and, if again the contaminated water is at or about the freezing point, they may survive for weeks together. In this way, the period of survival may be long enough. One should bear in mind, however, that there is no multiplication of the organisms in such a condition.

In some countries, people are accustomed to eat raw uncooked *oysters* which may harbour typhoid bacilli, particularly when the latter are collected from a place where sewage water flows. The bacilli may survive inside the oysters for nearly three weeks and then disappear, being killed or digested.

TOXIN PRODUCTION:—E. typhi does not secrete a true soluble toxin, i.e., there is no exotoxin. Toxic product of the organism is of the nature of an *endotoxin* and is produced either (i) by lysis of the bacterial bodies in the infected animal or (ii) by the action of ferments on the bacterial protein. The true nature of these endotoxins has, however, not yet been fully determined.

It should be noted that the specific toxins of E. typhi show a selective preference for the lymphoid tissues of the small intestine and spleen. As Eberthella typhi belongs to the pyogenic group of organisms, it may lead to pus formation at its site of lodgment.

In the endotoxin, a substance is present which, on intradermal injection into a rabbit, prepares the skin of the animal in such a way that an intravenous injection of the same material, 24 hours later, causes an acute purpuric and necrotic reaction at the site of the intradermal inoculation in 2 to 5 hours. This is what is known as *Shwartzman's phenomenon.* It should be noted that both the preparatory and the reacting agents are identical and are common to widely different bacterial groups, such as N. meningitidis, H. pertussis, etc. The reaction can, however, be neutralised specifically by an immune serum.

SEROLOGICAL REACTIONS:—When a broth culture of E. typhi is injected, in suitable doses, either intraperitoneally, or better still, intravenously, into a rabbit, certain antibodies appear in its blood after a week. These antibodies are mainly of the nature of agglutinins which tend to make the E. typhi lose their motility, come together and form clumps. These agglutinins were formerly considered to be specific for the typhoid organisms. A good deal of work has recently been done on this subject (*vide* p. 166) and many interesting observations have been made regarding the nature and qualities of such antibodies. For instance, the typhoid bacilli consist of two elements, the bacterial body (somatic endoplasm) and the flagellar elements. It has been found definitely that each of these elements forms a separate antigenic constituent and is capable of giving rise to the production of its own type of antibody in the serum of an inoculated rabbit.

The antigen consisting of the bacterial body has been called the O or S antigen or the endoplasmic antigen and the corresponding antibody which it produces is called the O or S agglutinin. These O agglutinOGENS are heat-stable and are not destroyed even at a temperature of 100°C. They produce very fine, compact granular masses in a Widal's test. These agglutinins are, however, very important for the production of active immunity and are responsible for the actual bacteriolytic phenomena. But they are not specific, as they will react to bacteria of allied species.

The antigen which consists of the flagellar elements has been termed the H antigen or the ectoplasmic antigen and its corresponding antibody is called the H agglutinin. These H agglutinOGENS are somewhat delicate, being destroyed easily at a temperature of 70°C. They produce large, loose, floccular masses which usually settle down at the bottom of a tube when doing a Widal's test. The H agglutinin is absolutely specific for the typhoid bacillus, but it does not play any very important rôle in the production of active immunity. In a naturally infected person or in one artificially inoculated with prophylactic vaccine, both the O and the H agglutinins are developed, although the latter develops in a much larger proportion.

ANIMAL INOCULATION:—Although E. typhi do not cause typhoid fever in laboratory animals (excepting the anthropoid apes), yet an injection of a living culture of the organisms, either into the peritoneal cavity or into the veins of these animals, causes septicæmia and death. A subcutaneous injection in sublethal doses causes a local abscess formation.

PATHOGENICITY

No animal, except man, suffers from typhoid fever.

Mode of infection:—Infection takes [illegible] from outside contagion. In most cases, there is usually a direct or [illegible] contact with a patient suffering from the disease or with a carrier. [illegible] illi gain entrance by way of the digestive tracts, through contaminat[illegible] and drink. *Flies* are very important in the transmission of the [illegible] as they may carry the bacilli and contaminate the above vehicles. [illegible] rriers (*vide* p. 423) also play an important part in the spread of the disease, particularly when they are employed in the kitchen or in a dairy farm. Thus the potent sources of infection are the following:—

(i) Water supplies contaminated with fæces containing the organisms.

(ii) Milk, milk products (ice cream) and other food stuffs contaminated by a carrier or flies which carry the germs mechanically from fæces to food.

(iii) Oysters, watercress and other uncooked vegetables (salads) which have been polluted with sewage contaminated by typhoid fæces.

Stomach acts as a barrier to infection and this is due to the presence of the hydrochloric acid in the gastric juice. If, however, the acid content is low, as happens when the stomach is empty or when the gastric juice is very much diluted by taking a large quantity of fluid, the bacilli escape the destructive effect of the latter and may pass on to the duodenum and jejunum where, in the alkaline medium, they can multiply. They then gain entrance into the mesenteric lymph nodes through the lymph follicles and Peyer's patches in the wall of the small intestine. From the latter they enter the general circulation via the lymph stream and are then carried all over the body. Thus, it is seen that *typhoid fever is primarily an infection of the lymphoid tissues of the small intestine, followed later by a condition of bacillæmia and still later, by a generalised infection.*

Course of the disease:—This can be described under the following stages:—

1. *Stage of infection or bacteriæmia*:—In this stage, the organisms multiply rapidly in the lymphoid tissues from which they are discharged into the circulation, producing a condition of bacteriæmia. During this process, many of the bacilli are killed, liberating their endotoxins which, however, must reach a sufficient concentration to produce the clinical effects of typhoid fever. This period which is called the *incubation period* is generally 2 to 3 weeks.

2. *Stage of localisation of the bacilli*:—As stated above, the particular tissue which is selected by the typhoid bacilli is the lymphoid tissue, specially that of the intestines, mesenteric lymph nodes and the spleen.

3. *Stage of development of immunity and healing of the lesions.*

4. *Stage of convalescence with associated complications.*

Lesions of *Eberthella typhi* may, for the sake of convenience, be described under the following heads:—

1. *At the site of entrance*, i.e., in the small intestine and mesenteric lymph nodes.
2. *Effects of generalisation* of the organisms and their ultimate localisation in the following organs:—
    (i) Biliary passages.
    (ii) Urinary system.
    (iii) Integumentary system.
    (iv) Osseous system.
    (v) Meninges.
3. *Effects of the toxins* on the muscular and hæmopoietic systems and other organs.

AT THE SITE OF ENTRANCE

Intestines:—The evolution of lesions in the intestine and their subsequent fate may be described in the following stages:—

Stage of hyperæmia and swelling of the lymphoid follicles:—It has already been mentioned that the organisms gain entrance through the lymphoid structures of the small intestine and multiply rapidly in the same locality. The endotoxins which are liberated by the death and disintegration of some of the organisms, produce marked vascular engorgement and hyperplasia of the lymphoid structures. As a result of this, they appear swollen and raised above the surrounding surface (Fig. 230). The cellular hyperplasia consists of an excessive proliferation of the small mononuclear cells which abound in these areas, together with an irritative hyperplasia of the endothelial cells of the capillaries and the cells of the reticulo-endothelial system. There is also a certain amount of catarrhal and necrotic changes in the superficial epithelium. The whole process takes about a week.

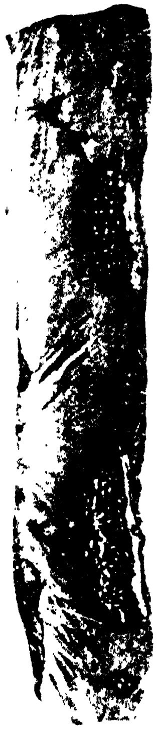


Fig. 230.
Early intestinal lesions in a case of *typhoid fever*, showing hyperplasia of Peyer's patches. Duration of the disease is about *a week*.

Stage of necrosis:—Degenerative changes, however, begin to appear very soon in these prominent lymphoid areas which undergo coagulative necrosis (Fig. 231). Such a change is primarily brought about by an alteration in the blood supply due to the obliteration of the lumen of the capillaries, either by thrombosis or by an excessive proliferation of the capillary endothelium.

Stage of sloughing and ulcer formation:—The necrotic mass now sloughs out with the formation of an ulcer (Fig. 232). According to the severity of the lesion, serious results may occur during the process of sloughing. At the time of separation of the sloughs, a blood vessel may be eroded, giving rise to a severe and sometimes fatal hæmorrhage (during the second to the fourth week) from the intestine.

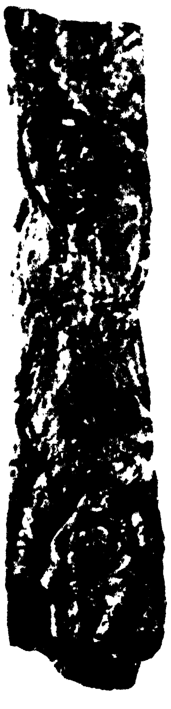


Fig. 231.
Intestinal lesions in the *second week of typhoid fever*, showing the stage of necrosis.

If the ulcer extends too deeply, reaching the peritoneum, it may cause either peritonitis or *perforation* (this occurs usually in the *third week*). It is to be noted that when perforation occurs, it is mo[illegible]n within the last one foot of the ileum, because it is in this part [illegible] the lymphoid elements, viz., the solitary lymph follicles and the Peyer's patches, are most numerous. Hence, this part of the gut has to bear the brunt of the disease. The stage of sloughing usually occupies about a week.

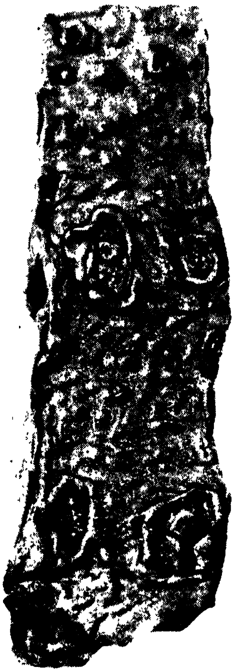


Fig. 232.
Intestinal lesions between the *second and the third week of typhoid fever,* showing the separation of slough and the formation of ulcer.

Stage of healing:—The ulcers, after remaining in this stage for varying periods, begin to heal up by the formation of granulation tissue. The mucous membrane, however, is restored completely so that, if such an intestine is examined later, no sign of previous ulceration could be recognised. This process is not associated with the formation of a cicatrix and there is hardly any stricture of the intestine due to the healing of a typhoid ulcer (*cf. tuberculous ulcer*).

Description of a typhoid ulcer:—

1. It is always in the small intestine (ileum), limited only to the lymphoid follicles and Peyer's patches.
2. Shape may be round (when the solitary follicles are affected) or oval (when the Peyer's patches are involved).
3. Long axis is longitudinal to the long axis of the bowel.
4. Situation is opposite the mesenteric attachment, because of the situation of the Peyer's patches.
5. Edges of the ulcers are raised above the surrounding mucous surface and moderately undermined.

   Base may show pitted or sieve-like appearance, or may be formed by muscular fibres or even serous coat only.

Difference between typhoid and tuberculous ulcers:—

	Typhoid ulcer.	**Tuberculous ulcer.**
Site	Small intestine; Peyer's patches and agminated lymph follicles affected.	Lymphoid tissues of small intestine; spread by lymphatics encircling the gut.
Shape	Round or oval.	Transverse.
Edges	Raised.	Undermined.
Base	Pitted appearance.	Mamillated.
Peritoneal surface	Nothing particular.	Small tubercles along the course of the lymphatics.
Perforation	Common.	Uncommon (fistulous communication between different coils of intestines may occur).
Matting of individual coils ...	Not present, unless there is peritonitis.	Present.
Healing ...	No sign is left behind; unassociated with stricture formation.	Marked scarring; associated with stricture formation.

Mesenteric lymph nodes:—These are always enlarged, swollen and congested. They are soft in consistency, the substance almost becoming diffluent in character. In extreme cases, they may suppurate and burst, causing peritonitis. Under the microscope, there is a marked dilatation of the lymph sinuses which are filled up by *mono-nuclear* and *endothelial cells*. The germinal follicles show marked hyperplasia with areas of focal necrosis scattered here and there.

Spleen:—This organ is always enlarged (acute splenic tumour) in typhoid fever. This is due to (i) a marked vascular engorgement and (ii) hyperplasia of the lymphoid and reticulo-endothelial cells. To the naked eye, the spleen looks enlarged and its capsule is tense. It is dirty red in colour and looks pulpy. It is very soft in consistency, the cut substance feeling almost diffluent. Large numbers of bacilli are found in this organ in all stages of the disease. *Microscopically*, the following features are found, such as congestion of the splenic sinusoids, proliferation of the lymphoid and reticulo-endothelial cells, large phagocytic mononuclear cells carrying red blood corpuscles in their cytoplasm and areas of *focal necrosis*.

EFFECTS OF GENERALISATION

Biliary passages:—Typhoid bacilli show a predilection for the gall bladder where they are of almost constant occurrence during the disease. They may set up a catarrhal or suppurative cholecystitis. There is evidence that the bacilli may persist for many years in the submucous coat of the gall bladder where they live and multiply and from which they are periodically discharged into the bile. In this way, the organ serves as a reservoir for the bacilli not only during the course of the disease but also afterwards, specially in the *fæcal carriers*. The presence of the bacilli in the submucous coat of the gall bladder keeps up a chronic catarrhal inflammation which may lead to the formation of biliary calculi. *Eberthella typhi* have been isolated from cases of gall stones operated on years after an attack of typhoid fever and the bacilli have been found alive within the calculi.

Nervous system:—The disease bears its name from the stuporous condition it produces. Actual cerebral changes, recognisable anatomically, are rare. Typhoid meningitis is very rare but, when such signs and symptoms occur, it is always due to a condition of hyperæmia and turgescence of the meninges (*meningism*), together with a fair amount of poisoning of the nerve cells (*encephalitis*). Cerebrospinal fluid, in such circumstances, is under pressure but clear. Isolation of *E. typhi* may be possible.

Respiratory system:—Typhoid bacilli may produce an initial bronchitis and the organism may be isolated from the sputum, but broncho-pneumonia and lobar pneumonia that may accompany typhoid fever, are all due to secondary infections. The organisms concerned are *Streptococci, D. pneumoniæ* and *other catarrhal organisms of the throat*. Broncho-pneumonia, as a complication of typhoid fever usually occurs from the end of the second to the fourth week.

Kidneys:—The kidneys are usually congested and show evidences of *toxic nephrosis. True nephritis is rare. Typhoid bacilluria* is fairly common; the bacilli continue to be excreted through the kidneys with or without producing inflammatory changes in the pelvis.

Bones and joints:—These are usually involved late in the disease or even a long time after. Localised periostitis of the tibia, ribs and other bones may take place. There is some involvement of the underlying bones, resulting in osteitis, osteoporosis and even suppuration and necrosis. In the discharge, typhoid bacilli may be constantly found. It is a very chronic process and takes a very long time to heal. Periostitis and osteo-periostitis of the spinal vertebræ is the cause of the so-called "*typhoid spine.*" The surrounding ligaments are more or less involved, resulting in pain on movement. A similar inflammatory process affecting the joints of the toes produces the condition commonly known as "*typhoid toe.*"

Integumentary system:—Rose spots are the characteristic rash of typhoid fever. These spots generally appear at the end of the first week and usually come in crops. They are seen most commonly on the abdomen. *Eberthella typhi* has been isolated from these rose spots.

Pyæmic abscesses:—Sometimes, during the course of a prolonged case of typhoid fever, particularly in its later stages, multiple abscesses may form in various parts of the body. Eberthella typhi has been isolated from such suppurative lesions. It may also be noted that such a condition may result from a *Staphylococcal infection* of the hair follicles.

EFFECT OF TOXINS

Muscular system:—The substance of the muscle fibres shows a sort of hyaline degeneration (*Zenker's degeneration*) which is not specific for typhoid fever but may be found in other acute infective processes. Common muscles affected are the abdominal, diaphragmatic and the adductors of the thighs. This degenerative condition in the muscles should always be remembered in connection with certain complications that may arise in the course of typhoid fever, such as broncho-pneumonia, persistent tympanitis and rupture of the rectus abdominis muscle.

Heart muscle:—Hyaline and granular degenerations may be detected in the muscle fibres. Such myocardial degeneration may account for a soft and dicrotic radial pulse.

Hæmopoietic system:—Leucopenia and anæmia in typhoid fever may be due to the paralysing action of the toxin on the bone-marrow. During complications, however, a moderate leucocytic response may be obtained from such a marrow.

Note:—Sometimes, during the late stages of the disease or during convalescence, *thrombosis of the iliac vein* (left side more commonly) may occur.

ROLE OF SECONDARY INFECTIONS

Various secondary organisms are responsible for certain complications which may arise during the course of typhoid fever. Thus, infection with *pyogenic cocci* may account for parotitis, broncho-pneumonia and pyæmic abscesses. Urinary complications, such as pyelitis and cystitis, may in some cases be due to *Escherichia coli* infection. In this connection, it should be noted that the invasion of *Streptococcus hæmolyticus* through the intestinal ulcers, resulting in the production of septicæmia, is based on an erroneous idea. Reference has already been made in the chapter on Streptococci that the type of cocci present in the intestinal tracts is *Streptococcus fæcalis* (*Enterococcus*) and it is this organism which may gain entrance into the blood stream. The pathogenic influence of such a condition in the course of typhoid fever is doubtful.

Carriers

After an attack of the disease, certain individuals remain as carriers and harbour the organisms either in the gall bladder, intestines or in the urinary tracts. Sometimes, a chronic enteritis or colitis persists and the stools in such cases show abundant bacilli. If an investigation is made, it will be found that about 11 to 12 per cent. of convalescent patients excrete the bacilli, either in their fæces or in their urine, for a period of 6 weeks or more, after the subsidence of the fever. These are classed as **temporary carriers.** A subsequent investigation of these temporary carriers will show that the majority of them have ceased to excrete the bacilli. Only 2 to 3 per cent. of cases excrete the bacilli for more than 6 months and thus become actual **chronic carriers,** probably for life. It will be noted that a period of 3 months is taken as an arbitrary standard and patients still excreting the bacilli after that time, are classed as chronic carriers. These may be of two types, fæcal and urinary.

(1) The percentage of *fæcal carriers* is about 2.5 per cent. and the focus of infection in such cases is mostly in the gall bladder and rarely in the intestine. It is in the submucous coat of the gall bladder that bacilli inhabit and multiply, producing mild inflammatory lesions. The organisms are discharged intermittently into the bile and then into the fæces. These are otherwise fit men but complain, from time to time, of pain over the gall bladder region with fever. In many of these cases, this continued infection of the gall bladder has resulted in the formation of gall stones which eventually call for surgical interference.

(2) *Urinary carrier*—In 0·5 per cent. of the cases, the condition of typhoid bacilluria persists for more than 2 months after defervescence, sometimes very much longer, and thus helps in the dissemination of the disease. The site of infection is the pelvis of the kidney. In long standing cases, there is often an associated cystitis with pyuria.

Detection of carriers:—The ultimate diagnosis of such persons will depend on the *isolation of the bacillus* from the fæces, urine or duodenal contents or from the pus from a bone abscess. The cases are selected in the following ways:—

1. *On epidemiological grounds*—The source of an epidemic is investigated and it is found that either a cook, or a man employed in the dairy farm, is responsible for the dissemination of the disease.

2. *Widal reaction*—This provides another method of selection. It is now generally held that those who give a marked positive reaction six months after the fever, are very probably carriers. It should be noted that although the reaction is usually positive but it is not invariably so in carriers.

3. *Skin reaction* (McKendrick)—A positive reaction is obtained in persons who are supposed to be chronic carriers. In order to perform the test, about 0·04 to 0·08 c.c. of a standardised bacterial emulsion is injected intradermally. The reaction is said to be positive when a swollen indurated brownish papule with an extensive areola appears at the site of the inoculation on the third to the fourth day.

4. Lastly a previous history of having had the disease and of recurring pain and tenderness in the gall bladder region is occasionally helpful.

After the selection of cases, the bacteriological examination of the material collected is carried out in the usual way (*vide infra*). In collecting a sample of fæces, it is advisable to give the person a preliminary saline purge (magnesium sulphate) preceded by a dose of calomel. This helps to increase the biliary flow, thereby bringing the organisms from their abode to the intestine.

Treatment of carriers:—This is very important from the public health standpoint, as this is one of the surest measures in the prevention of typhoid fever. Various methods, such as intestinal, biliary and urinary antiseptics and vaccination have been tried, but to no effect. Removal of the gall bladder (cholecystectomy), however, appeared to have cured a considerable number of fæcal carriers. The wisest course in such cases, therefore, is to keep these chronic carriers under observation so that they may not have any chance to contaminate the water supply or to handle food stuffs.

THERAPEUTIC CONSIDERATIONS

These may be considered under the following heads:—

(1) ***Curative:***—

(a) Although E. typhi gives rise to the production of a high concentration of agglutinins in the blood of animals and man when injected, nevertheless such a **serum** is neither antibacterial nor antitoxic, when used in an actual case of enteric fever. Such a *serum,* therefore, has no curative effect and its use is futile to control an infection.

(b) Similarly, the administration of a **typhoid vaccine** for the control and cure of an infection has been found to be equally disappointing. It is also useless to try to cure the chronic carriers with vaccine therapy.

(c) **Typhoid bacteriophage:**—Sometimes, in the early days of the disease, bacteriophage is employed in the usual way. But the efficacy of such a treatment is doubtful, as it is known that the disease is no longer a local one but a bacteriæmia with secondary localisation. It is given in doses of 2 c.c. every four hours for 5 or 6 days.

(2) ***Prophylactic:—***

(a) **Typhoid paratyphoid vaccine** (*commonly called T.A.B. vaccine*): —When we come to consider its prophylactic use, we find a marked difference. The development of agglutinins and the amount of immunity conferred against an infection has been found to be so striking that the use of the vaccine has been regarded as a recognised method in prophylaxis. We owe to *Sir Almoth Wright* and his colleague *Sir W. Leishman* for introducing the vaccine inoculation with its immense immunising value in the prophylaxis of typhoid fever. The method was not so widely accepted until its introduction amongst the fighting population in the Great European War, which definitely proved its advantages in reducing both the incidence and severity of enteric infection and placed it on a solid basis. Thus, to take concrete examples, reference should be made to Greenwood and Yule's figure (1915), who showed that out of 10,378 inoculated persons, the incidence of the disease was only 56, whereas amongst 8936 non-inoculated persons, 272 were attacked, i.e., about 5 times more. Similarly, Pfeiffer (1922) has shown that the case mortality amongst the German army was reduced from 12 to 30 per cent. in the non-inoculated to about 2 to 3 per cent. in the inoculated.

Dosage and Methods of Administration:—The vaccine which is most commonly employed for this purpose is the one prepared from the *smooth type* of E. typhi (B. typhosus), S. paratyphi (B. paratyphosus A) and S. schottmülleri (B. paratyphosus B). A young 24 hours' culture on agar is emulsified in saline and sterilised by heating it in a water bath at 60°C. for one hour; 0·5 per cent. carbolic acid being used as a preservative. The dose is so regulated that 1 c.c. of the vaccine contains 1,000 millions of E. typhi (B. typhosus) and 500 millions each of S. paratyphi and S. schottmülleri (B. paratyphosus A and B). About 0·5 c.c. of the emulsion is injected subcutaneously as the initial dose, followed about 8 to 10 days later by another injection of 1 c.c. Usually this is enough, but a third injection of 1 c.c., another week later, gives a thorough protection and should be advocated whenever possible.

Reaction:—Usually, after such a prophylactic injection, the inoculated person feels some pain and tenderness locally and malaise and feverishness within 6 to 8 hours. In some cases, however, the fever may be high and attended with chill and rigor. There is swelling, erythema and œdema at the site of inoculation and some tenderness of the proximal lymph nodes. The whole condition lasts from 24 to 36 hours, after which both the local and the general conditions rapidly improve. On the whole, the reaction is never so alarming and so far there is no record of any serious accident after its inoculation.

Development of Agglutinins:—After a fu [illegible] of injections, the blood serum of the individual should be tested for t [illegible] unt of agglutinins developed. It is usual to find the titre limit for E [illegible] (B. typhosus) to be 1 in

1,000 or above. The titre limits for the other two organisms, i.e., S. paratyphi and S. schottmülleri (B. paratyphosus A and B) reach 1 in 500 or above. In this respect, it should be remembered that individuals vary widely in their responsiveness to antibody formation and for this reason the titre limits cannot be placed at any definite level.

Nature of Agglutinins:—When antityphoid inoculation is made, both the flagellar or H and somatic or O agglutinins are developed in the serum of the inoculated person. But the formation of H agglutinin is the most marked and its titre reaches a very much higher limit. This H agglutinin is demonstrated as floccular masses which are sent down at the bottom of the tube in doing a Widal's test. The somatic or O agglutinin which produces fine granular precipitates, forms in very small concentration which hardly reaches a titre limit of 1 in 100. Although the latter is present in the serum in such a low dilution, nevertheless, most of the protective power depends on it rather than on the H agglutinin.

How long does the effect of inoculation last?

Normally, the agglutination titre reaches its maximum height from the sixteenth to the twentieth day after which it progressively falls till the eighth week. After this period it remains in the serum in demonstrable quantities for months and even years. This small amount of antibody together with an increased reactivity of the body cells is enough to protect the inoculated individual from an infection. The protective power lasts definitely for about a year but fresh inoculations should be undertaken after about one and a half years, if the risk of infection be present.

SOME PRECAUTIONS:—Usually, after the inoculation of a T.A.B. prophylactic vaccine, during the first 5 to 6 days, there is no response; in fact, the resources of the body are somewhat exhausted. This period is commonly called the *negative phase* (*vide* p. 144). After this time, however, the responsiveness of the tissues progressively increases and this is called the *positive phase*. This will explain why the second dose of the vaccine should never be given earlier than a week. It is for this reason also that before inoculation, one should see that the patient is in a satisfactory state of health. Any ill-health or constitutional depression should form a contra-indication to prophylactic inoculation. For the same reason, it is sometimes undesirable to inoculate a person who has already been exposed to infection or who may be in the incubation period of the disease.

In the preparation of the vaccine for prophylactic inoculation, the selection of the strain is of utmost importance. The strain must be of the *smooth* type and also *highly motile*. It should be highly agglutinogenic. Unless these points are carefully attended to, the prophylactic vaccination is bound to result in a failure.

(b) **Oral Vaccination:**—Although the majority of the European and American schools believe in the formation of specific antibodies in the blood and tissues for conferring protection on the individual, a new idea has become current lately, owing to the interesting theory advocated by Besredka. According to him, the resistance to an attack by E. typhi (B. typhosus) depends entirely on the mucous membrane of the small intestine and if an individual suffers from an attack, it is because the intestinal mucosa fails to stop the progress of the bacilli. Thus, according to him, by raising the local immunity of the intestinal mucous membrane, the general infection may be avoided. Furthermore, he found that the administration of bile, as a preliminary measure, considerably helps in the production of such an immunity. With this idea, he has introduced oral vaccination as a prophylactic method and the ingredient used is a combination of dead bacilli with ox bile and is known as bilivaccine.

Prophylaxis:—

1. Detection and isolation of the cases.

2. Disinfection of their excreta, beddings, wearing apparels and the hands of attendants. The measures adopted for disinfection are the following:—

   (a) Perchloride of mercury for washing hands.

   (b) Boiling and exposure to sunlight for clothings.

   (c) Cresol and other coal tar derivatives for treating the sewage.

   (d) Chlorination of drinking water during an epidemic. During such periods, drinking of boiled water forms an additional safeguard.

3. Prophylactic inoculation with T.A.B. vaccine.

LABORATORY DIAGNOSIS

The laboratory diagnosis of typhoid fever rests on two factors:—

(i) Isolation of the organism from the body of the patient and its identification (*bacteriological diagnosis*).

(ii) Serological changes, produced as a result of the infection with Eberthella typhi (*serological diagnosis*).

Isolation of Eberthella typhi (*B. typhosus*):—The organism is usually isolated from the blood, fæces and urine. But it may also be recovered from the rose spots on the skin, cerebrospinal fluid, sputum, bile and pus in suppurative conditions produced by E. typhi.

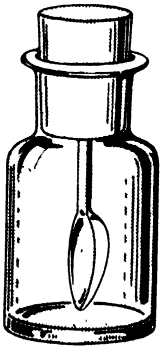


Fig. 233.
Container for fæces.

Collection of material:—

Blood—This is usually taken from one of the superficial veins in the anticubital fossa. Strict asepsis should be observed during this procedure.

Fæces—The best way to collect the material is to take it in a fæces tube, as shown in Fig. 233.

Urine—Catheter specimens should always be preferred. In the case of the [illegible], however, the mid-stream urine may be taken.

Detection of the organism:—Isolation is usually done in one of the special media, the most widely employed being the MacConkey's plate. The organism is recognised by the following characters:—

1. *Non-lactose fermenter.*
2. *Formation of acid but no gas in glucose broth.*
3. *Active motility.*

The identity of the organism is then confirmed by the following steps:—

(a) Determination of its biochemical reactions, such as its action on the litmus milk and other sugars.

(b) Its agglutinability to specific sera—This is of the greatest assistance in the identification of a suspected bacillus, as E. typhi. For this purpose a high titre serum (1 in 2,000 or upwards), obtained by immunising rabbits with a known typhoid organism, is kept in the laboratory in an ice chest. The test is put up with suspensions of the organism from young broth cultures or preferably, from an emulsion of young agar culture in normal saline. The actual test is carried out either by the "slide method" or by the use of the usual agglutination tubes. If the known high titre serum agglutinates the organism to nearly its full titre, the presumption is that the new bacillus is E. typhi. It is of importance to note that occasionally a true typhoid bacillus, when freshly isolated from the body, is relatively, or even totally, inagglutinable. But usually, it becomes agglutinable after being subcultured a few times in the laboratory.

Some points about the cultural examination of the blood, stool and urine:—

Blood culture—The possibility of a successful recovery of the organism from the blood depends upon the period of the disease in which the examination is done. The most favourable time is the early phase of the infection, i.e., at the stage of bacteriæmia which is usually marked during the first five days. The chances of success, however, diminish rapidly after the first week, as shown in the following table (Fig. 234):—

First week	95 per cent. positive.
Second week	70 per cent. positive.
Third week	35 per cent. positive.
Fourth week	10 per cent. positive.
Fifth week	2 per cent. positive.

For a successful result, the following methods should be followed:—Five to ten cubic centimetres of blood should be withdrawn aseptically from a vein, preferably at the bend of the elbow, and transferred immediately under sterile precautions to a flask containing 100 c.c. of nutrient broth, either with one per cent. glucose or half to one per cent. sodium taurocholate. The enrichment with the last two reagents is not absolutely necessary but helpful.

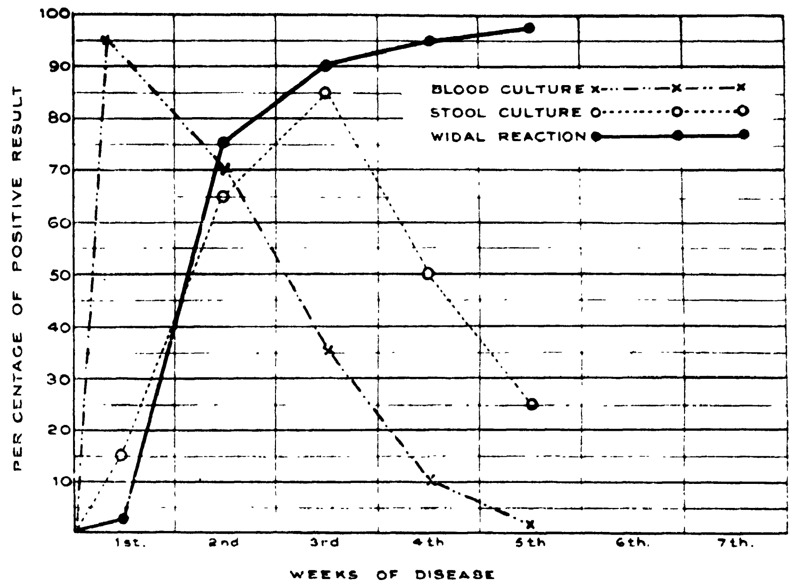


Fig. 234.
Chart showing the relative importance of blood cultures, stool cultures and Widal reactions in different weeks of *typhoid fever*.

Another good method is to put the blood into a tube containing fresh sterilised ox-bile. The flask or the bile tube is incubated for 24 to 48 hours, after which it may be examined for any growth. Sometimes, the growth of the organism may be delayed and one has to wait for 4 to 7 days before pronouncing it as negative. If it is positive, one should find, under the ordinary high power of a microscope, highly motile bacilli. Subcultures are then made on agar and special selective media, such as MacConkey's plates. After 24 hours' incubation, the characteristic colonies may be noted and further studies made regarding sugar reactions and agglutination tests with specific high titre serum.

If the patient stays at a distance from the laboratory and the inoculated culture media have to be carried, there is always a risk of contamination, as the cotton plug of the flask gets soaked during the transit through jolting and violent agitation. Under such circumstances, it is always safe to transfer the blood into a long test tube, containing about 10 c.c. of sterilised 2 per cent. sodium citrate solution in normal saline. The blood is thoroughly mixed with this citrated saline by rapid rotary movements, after which it may be kept inside the bag and carried safely to the laboratory where necessary inoculation may be made conveniently.

Transport by parcel may be facilitated by d g the blood in a special Behring's venule, containing either sterile citrat ne or ox-bile or glucose broth.

Note:—(i) Many failures are due to inocu with insufficient quantities of blood. It should always be about 10 c on no account, be less than 5 c.c.

(ii) Whenever blood is taken for cultural examination, it is always best to take more than what is necessary, so that a portion of it may be collected in a dry sterile test tube and kept apart for doing an agglutination test at the same time.

(iii) The clot which is left after collecting the serum from Wright's capsule may also be utilised for making cultures (*clot culture*).

A few words about the nature of contamination will not be out of place. The commonest contaminating organism is the *Staphylococcus* which comes in either from the skin surface or from the atmosphere. But the one which may mislead the pathologist is *Bacillus subtilis*, the spores of which are very resistant and present in the air, dust and imperfectly sterilised glass vessels. It is a very highly motile organism and therefore adds more to the confusion. But before depending on its morphology and motility only, one should do a Gram's stain which will settle the diagnosis at once; for, whereas the *Eberthella typhi is Gram-negative*, Bacillus subtilis is Gram-positive.

Stool culture:—The chances of success in obtaining a positive result from the stool examination are shown in the table (*vide* Fig. 234):—

First week	15 per cent. positive.
Second week	65 per cent. positive.
Third week	85 per cent. positive.
Fourth week	50 per cent. positive.
Fifth week	25 per cent. positive.

Isolation of E. typhi from the stool is a very important step in the practical diagnosis of typhoid fever. Although the bacilli may be present in the stool from the first week till complete convalescence or even some weeks later (the latter condition often happens in cases which turn out to be carriers), the period when they are excreted in largest numbers is from the *second* to the *fourth week*. In the case of carriers, however, the discharge of the bacilli is often intermittent, there being some periods when they are entirely absent and others when they are excreted in very large numbers.

It is very important for physicians and pathologists to remember some points when they want to undertake an examination of samples of stool suspected to contain *E. typhi*. Failure to remember them often ends in negative results and is the source of much misunderstanding between the physician and the bacteriologist. The most important factor is the *freshness of the stool*. After passing, if the stool is kept for some time, specially in a tropical country like ours, the typhoid organisms are rapidly overgrown and often killed by Escherichia coli and other vigorously growing saprophytic bacteria. It is necessary, therefore, that the specimen of stool be available for examination immediately after it is passed. The delay of even a few hours in this respect is harmful. To ensure this point, the physician concerned should leave written instructions to the nurse and attendants about the collection and despatch of the stool. If the patient resides at a distance so that the transit of the material takes a long time, or if the examination cannot be undertaken immediately, it is always best to mix a little stool with double its quantity of a solution containing 30 per cent. glycerin in 0·6 per cent. saline. In such a mixture, the bacilli have been found to keep well and for a long period of time.

In culturing the stool, it should be remembered that Escherichia coli and other saprophytic organisms grow much more vigorously than Eberthella typhi whose growth is thus somewhat inhibited. For this reason, it is always advisable to use, if possible, culture media enriched with reagents which have been found to inhibit the growth of bacteria other than E. typhi, leaving the latter to grow unhampered. The two substances which are specially helpful in this respect are brilliant green and telluric acid. A loopful of the suspected stool is thoroughly emulsified in peptone water containing the above two substances (*vide* page 69). After waiting for a *little while*, a MacConkey's plate is inoculated with a drop of the supernatant fluid. Both the tube and the plate are then incubated for 24 hours. After this time, the plate is examined and if colonies of non-lactose fermenting organisms are found, the latter are subjected to further studies regarding motility, sugar reactions and agglutination tests and the examination of the brilliant green tubes need not be proceeded with. But if no non-lactose fermenting colonies are detected in the plate, fresh plating should be made from the brilliant green tube at 24 hours' intervals, successively for 3 or 4 days. If the examination is negative, one should wait for 3 to 4 days and then undertake another examination and in this way, the examination should be repeated several times. A stool which is found to be negative during one examination may be found to show large numbers of typhoid organisms at a subsequent culture.

Urine culture:—The elimination of the organism in the urine is more intermittent and is of a shorter duration (*transient bacilluria*). In the case of the urine, a quantity of the material collected aseptically should be mixed with about 3 times its volume of brilliant green peptone water or nutrient broth with the same dye. In this case, however, ordinary nutrient broth without any enriching substance will serve the purpose quite well as there is often no question of other secondary organisms, as in the stool. The subsequent stages of the examination are the same as in the case of the examination of the stool.

Serum diagnosis:—For collection of the serum and the technique *vide Chaper XIV.*

In the early days of the disease, it is not [illegible]le to demonstrate the presence of agglutinins in the blood serum. Bu[illegible] the first week, appreciable amounts of agglutinins appear in the bloo[illegible] titre limit gradually increases, until it reaches its maximum on the twenty-first day (Fig. 235). Afer this period, it declines very slowly, but may occasionally persist for some years. Hence, the best period in which a positive diagnosis can be obtained from Widal's reaction is after the first week (10 days) of the disease (vide Fig. 234) as shown in the following table :—

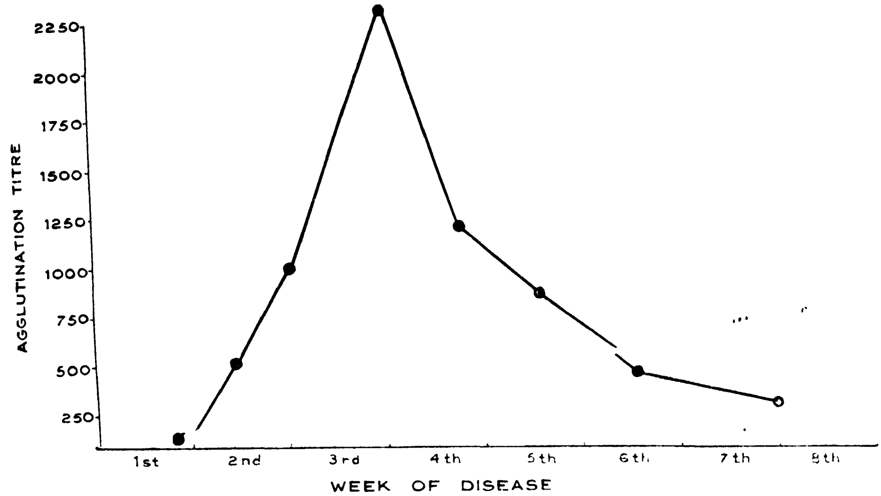


Fig. 235.

Agglutinin response in a case of *typhoid fever*.

First week	...	...	...	3 per cent. positive.
Second week	...	...	...	75 per cent. positive.
Third week	...	...	...	90 per cent. positive.
Fourth week	...	...	...	95 per cent. positive.
Fifth week	...	...	...	97 per cent. positive.

As already mentioned, the infection with Eberthella typhi evokes two types of agglutinin response—H or flagellar and O or somatic agglutinins. For ordinary routine diagnosis, H agglutination suffices, as this appears to be specific. But in some rare cases, H agglutinins fail to appear in the serum, particularly if there be infection with a non-motile strain (Felix). It is in these cases that examination for O agglutinins is necessary for which an alcoholised suspension should be used as Dreyer's formolised suspensions do not react with O agglutinin. Sometimes, it happens that the agglutinins appear quite late in the disease or may not appear at all. The prognosis in these cases is not favourable, for reference has already been made that there is some relation between immunity and somatic or O agglutinins (*vide* p. 168).

Diagnostic standard :—

(A) *Infection in the non-vaccinated persons:*—With E. typhi and Salmonella schottmülleri (B. paratyphosus B), the lowest titre of the serum for H agglutination which should be accepted as diagnostic is a dilution of 1 in 50. During the height of the infection, i.e., second to third week, it is the rule, however, to obtain a positive result in much higher dilutions of the serum. With Salmonella paratyphi (B. paratyphosus A), the lowest diagnostic limit should be a dilution of 1 in 25, because the agglutinability of this organism is less marked than that of the E. typhi and S. schottmülleri.

(B) *Infection in the vaccinated persons:*—As already mentioned on *page* 168 that when a person is inoculated with T. A. B. vaccine, a marked response of H agglutinins is obtained and, though after some time it disappears, any non-specific stimulus may raise the reaction to a very high titre (anamnaestic reaction). Hence, a great difficulty is experienced in interpreting the results of the Widal reaction (H agglutination) in these cases. A rising titre, however, is in favour of a diagnosis of the infection. But by relying on the somatic agglutination, such difficulties can be overcome, as O agglutinins are produced by the specific stimulus alone and O titre of 1 in 100 is practically diagnostic of the present infection. Reference has already been made that O agglutination is a group reaction and therefore signifies that the infection is due to the enteric group of organisms.

It should be remembered that the classical Widal's test fails in three directions:—

(i) In the early serological diagnosis of typhoid fever.
(ii) In the previously inoculated individual.
(iii) In the persistently negative cases, i.e., infection with a non-motile strain.

In all these cases, O agglutination will settle the diagnosis. Hence, for a serological diagnosis of typhoid fever, both H and O agglutinations should be carried out in every case.

To *conclude*, the bacteriological diagnosis of typhoid fever may be summed up as follows:—

Of the disease:—

First 7 to 10 days	...	By blood culture.
After the tenth day	...	By Widal reaction.
At all stages but particularly after the second week	...	By stool and urine culture and by Widal reaction. The diagnostic value depends on a rising titre.

Of the relapse: Always make a fresh blood culture in the case of a relapse. Even if it was negative before, it invariably yields a positive result during the relapse.

Of the carriers:—

(a) By the cultivation of a sample of bile, fæces or urine.
(b) By Widal's reaction.

Post mortem diagnosis:—This consists in isolating the organism from the heart's blood, gall-bladder bile, splenic pu[illegible]senteric lymph nodes, scrapings from the intestinal lymphoid tiss[illegible]rs) and fæces. The chances of success depend on the interval whi[illegible]ses between the death of the patient and the post mortem examinati[illegible]he materials from the organs are collected with a sterile pipette, fitted [illegible] teat, plunged through an area on the surface of the organ sterilised b[illegible]hot searing spatula.

DYSENTERY GROUP OF BACILLI

HISTORICAL:—In the year 1898, *Kiyoshi Shiga* isolated an organism from the fæces of patients suffering from acute dysentery in Japan. Two years later, *Kruse* (1900) isolated a similar bacillus in Germany; hence, this type of dysentery bacillus is called the Shiga-Kruse type. Later on, *Strong* in Manila and *Flexner* (1900) in the Philippine islands isolated other strains of dysentery bacilli which were found to differ in certain biochemical and serological properties from the Shiga-Kruse type. Although the different strains have been isolated from different epidemics, all of them possess some common characters which bring them under this group. The production of a soluble extra-cellular toxin by the Shiga strain was noted by *C. Todd* (1903, 1904).

Characters of the group as a whole:—

1. *Belong to the Eberthella group.*
2. *Non-motile and devoid of any flagellum.*
3. *Gram-negative.*
4. *Non-lactose fermenters.*
5. *Ferment glucose with production of acid only: all of them are non-gas producers.*

VARIETIES:—There are several strains of dysentery bacilli which can be grouped into two main divisions. These differ in their behaviour towards certain sugars (mannite), in their capacity to form indol and in their agglutination reactions.

They are as follows:—

1. ***Shiga-Kruse type:***—The organisms of this group do not ferment mannite, nor form any indol and are serologically distinct but homogeneous, e.g., *Eberthella dysenteriæ* (B. dysenteriæ, Shiga).

2. ***Flexner type:***—The organisms of this group ferment mannite and form indol, but serologically form a heterogeneous group, e.g., *Eberthella paradysenteriæ* (B. dysenteriæ, Flexner).

MORPHOLOGY AND STAINING:—These are the same as mentioned under general characters of the Gram-negative intestinal bacteria with the exception that they are non-flagellated and devoid of any motility. They possess, however, a very active Brownian movement.

CULTURAL CHARACTERS:—They are aerobic but facultatively anaerobic. These organisms grow well in a reaction of pH7·5. The dysentery organisms are highly sensitive to acid reaction. A slight acidity tends to inhibit, whereas alkalinity favours, their growth. This is particularly so with E. dysenteriæ which can grow even in a reaction of pH11·0. They grow easily in ordinary as well as in special selective media, like the other intestinal pathogens. The colonies (being non-lactose fermenters) resemble those of E. typhi but they are somewhat more delicate.

BIOCHEMICAL REACTIONS:—

Carbohydrates:—Of all the carbohydrates, the fermentation reactions in glucose, lactose and mannite have been found to be constant. None of the dysentery organisms, however, produce gas in any sugar medium.

Lactose—None of the strains produce any change in lactose.

Glucose—All the strains ferment it, producing acid only.

Mannite—Some strains ferment mannite while others do not, e.g., Shiga's strain does not ferment mannite, whereas Flexner's group does.

Maltose, saccharose and *dulcite* are also used for differentiation of the individual strains.

Action on litmus milk:—During the first few days it becomes faintly acid without any clotting. Later, the reaction may change and revert to neutrality and even to an alkaline side, due to its proteolytic effect.

Action on proteins:—

Formation of indol:— E. dysenteriæ, Shiga never produces indol which, however, is formed by the Flexner group.

Formation of volatile fatty acids:—It has been found that the growth of the organisms in peptone water results in the production of a number of fatty acids, such as acetic, formic and butyric acids and also in the lowering of the pH. Such volatile fatty acids, produced in large amounts in the intestinal tracts of a person suffering from dysentery, may play an important part in the digestive disturbances and toxic symptoms accompanying the infection caused by them.

RESISTANCE:—These organisms cannot withstand the action of heat, light, desiccation and chemical disinfectants more than the other pathogenic members of the intestinal group. They are readily killed by a temperature of 60°C. in 10 minutes. But while this is true of cultures in artificial media, the conditions are somewhat different under natural circumstances. Under suitable conditions of temperature, moisture and oxygen supply, the organisms may survive for a considerable period in infected soil, water and fomites. It is very important to bear this fact in mind during the study of epidemic outbreaks of the disease.

TOXIN PRODUCTION:—**Eberthella dysenteriæ** *(B. dysenteriæ, Shiga)* produces two kinds of toxins as shown below:—

(i) An *exotoxin* having a specific action on the central nervous system, causing paralysis of muscles. This is called "*parietic*" toxin which produces no effect on guinea-pigs, although rabbits are affected by it. This "*parietic*" toxin is neutralised by an antitoxin and is destroyed by heating to 81°C. for one hour.

(ii) An *endotoxin*, having a specific affinity for the intestines, causing diarrhœa, chronic marasmus and fall in temperature. This is called "*marasmic*" or "*intestinal*" toxin and affects both gui[illegible]gs and rabbits. It is not neutralised by antitoxin.

Eberthella paradysenteriæ *(B. dysenteriæ, Flexner)* does not produce any soluble exotoxin. It has an endotoxin which is liberated only on the death and disintegration of the bacterial body. This is less toxic and requires a dose about 20 times larger than the corresponding lethal dose of Shiga's strain to prove fatal.

SEROLOGICAL REACTIONS:—Agglutinins are found in the serum of immunised animals and patients suffering from the disease and this may be utilised for diagnostic purposes. Appearance of agglutinins becomes most marked towards the end of the first week in acute cases. In chronic cases, however, it may be much delayed and also less marked. It should be remembered that as the bacilli are non-motile, only O-type of agglutinins are found.

It has been further observed that serologically E. dysenteriæ, Shiga forms a distinct homogeneous group, all strains of the organism being agglutinated by a specific antiserum prepared against any one of the Shiga strains. There is, however, some development of group agglutinins, as a result of which an anti-Shiga serum may agglutinate, to some extent, the Schmitz bacillus and some varieties of the Flexner group of organisms. It may be noted in this connection that the limits of titre of such group agglutinins are usually low. The mannite fermenting paradysenteric organisms, on the other hand, form a heterogeneous group, so that an antiserum prepared against any one particular strain either fails to agglutinate all the other strains or does so at low and irregular titres. This irregularity in their serological reactions has been explained in the light of modern researches by the varying antigenic constituents of these organisms. Andrewes and Inman have definitely proved that the Flexner's bacilli are composed of at least four different antigenic components (V, W, X and Z), each of which may be found, to a variable extent, in every strain, although in any given strain, 'there is usually some preponderance of one antigen over the rest.' In this way, they have been able to demonstrate that the Flexner group consists of at least 5 distinct races and they are named V, W, X, Y and Z. The Y strain is a composite mixture of the four races in approximately equal proportions and a serum prepared against this has a wider agglutination range, reacting with all the Flexner group of dysentery bacilli.

ANIMAL INOCULATION:—No very characteristic lesions are produced in animals by the administration of dysentery bacilli or their toxins *per os*. In rabbits, intravenous injection of Shiga toxin causes hæmorrhagic enteritis with membrane formation and ulceration. If the animal lives long enough, paralysis of the hind-limbs occurs as well. Flexner's group of bacilli is less pathogenic to rabbits.

PATHOGENICITY

This group of organisms produces an acute inflammation of the large intestine (acute colitis), characterised clinically by the passage of blood and mucus in the stool (dysentery), associated with tenesmus and griping.

Mode of infection:—Dysentery may be conveyed by different agencies, but the infection is mostly transmitted from man to man indirectly by contaminated *food stuffs*. The chief element in the spread of the disease is the "*carrier.*" These men are specially dangerous when they are employed in the dairy farm and kitchen. What usually happens is that they suffer from mild intestinal disturbances to which they themselves pay very little attention, but which form a fruitful source of scattering the infectious material and spreading the disease. Food may also be contaminated through the intervention of *flies* and this mode of transmission is of the utmost importance in epidemic outbreaks of dysentery. Whether or not domestic animals act as carriers is not certain but it has been suggested that dogs may be spontaneously infected and suffer from bacillary dysentery and transmit the disease to human beings.

Portal of entry: —From the above, it will be seen that the organisms gain entrance into the alimentary canal by the *ingestion* of infected foods. The bacilli multiply in the large intestine and remain strictly localised in the bowel. They never invade the blood stream, i.e., there is *no bacteriæmia;* only in extreme cases, they may go up to the mesenteric lymph nodes. Multiplication of the bacilli in the intestine results in the formation of a large amount of toxins (*vide* p. 435) which are responsible for the production of the various lesions in bacillary dysentery.

Pathogenic lesions

Intestine--The chief lesion is found in the intestinal tract, particularly in the large bowel. The lesions are found more commonly in the lower part of the large intestine and only very rarely, in the lower few inches of the ileum. The bacilli rapidly multiply in the intestinal canal and produce a toxin which is absorbed and gives rise to an intense inflammation of the mucous membrane of the large bowel. The most important features of the lesions (Fig. 236) are the following:---

(1) *Intense hyperæmia and even hæmorrhages in the mucous and submucous coats.*

(2) *Inflammatory œdema of the submucosa with extensive round-cell infiltration.*

(3) *Coagulative necrosis of the epithelium.*

(4) *Deposition of a large amount of fibrin on the surface.*

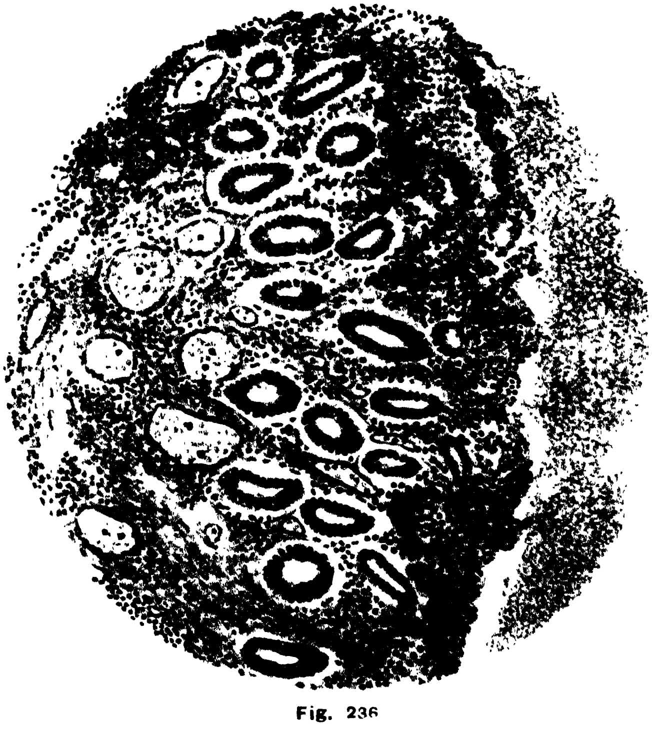


Fig. 236

Photomicrograph of a section of la[illegible]ine in acute bacillary dysentery, showing engorgement of [illegible] vessels and leucocytic infiltrations in the mucous and su[illegible] coats. There is also formation of a fa[illegible]ane.

The deposition of fibrin is seen, in the earlier stages, over the prominent folds and rugæ of the mucous membrane, but soon the entire surface becomes covered with a sheet of inflammatory exudate (Fig. 237). Along with this,

Fig. 237.

Large intestine from a case of acute bacillary dysentery (Shiga infection), showing formation of diphtheritic membranes (coagulative necrosis) which are most marked over the folds of the mucosa. Note the characteristic green colouration of the necrosed mucous membrane and rose pink colour of the intervening portions.

there is a continuous outpouring of an inflammatory fluid, rich in coagulable lymph and containing many pus cells, red cells and large phagocytic mononuclear cells (macrophages) into the lumen of the gut. This fluid comes out during motions, producing the characteristic evacuations of bacillary dysentery in the acute stage.

Ulcer formation:—Later on, the fibrinous deposit is cast off and ulcers develop. These are at first superficial, involving the mucous and submucous layers, and are distributed all over the mucous surface as sinuous, snail-track or serpiginous ulcerations (Fig. 238). They may communicate with one another by submucous sinuses. Their surfaces are covered with greenish black sloughs. The entire wall of the bowel is much thickened, due to an inflammatory œdema and cellular infiltration.

Gangrene of the bowel in debilitated persons:—Sometimes, particularly in patients suffering from debility and chronic malnutrition, the inflammation may be so very acute that it culminates in gangrene of the wall of the entire colon.

Local peritonitis due to secondary infection by Escherichia coli and Streptococci may be produced, particularly when the ulcerations are deep.

Discharge of intestinal casts:—Occasionally, all the inflammatory exudates together with the necrosed mucous membrane are cast off into the lumen and passed out with the fæces in the form of a tube. These are the so-called intestinal "casts" which often cause much alarm to the patient and his attendants.

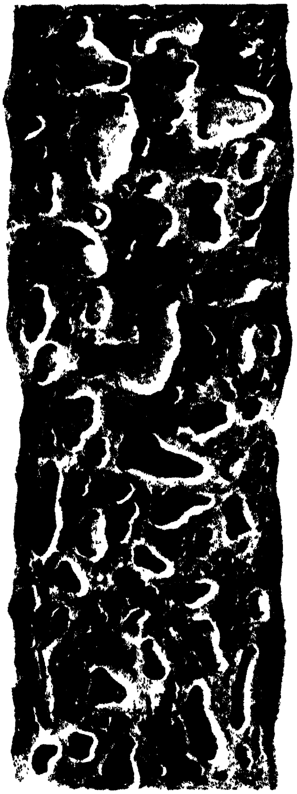


Fig. 238.

Ulceration in the large intestine of a case of bacillary dysentery. Note the serpiginous character of the ulcers. There is practically no healthy mucous membrane in between the ulcers.

Resolution:—If conditions are favourable, resolution may take place. The œdema and inflammation subside, the exudate is partly shred and partly absorbed and the mucous membrane restored to its normal condition. Cases with marked ulcerations have a different termination. When the ulcers are very deep and involve wide areas of the mucosa, they usually heal by granulation. Such a healing process is [illegible]npanied by much fibrosis and pigme[illegible]. The epithelium does not grow ove[illegible] cicatrices which may thus be de[illegible]uring a necropsy examination long afterwards as pigmented scars. It [illegible]o find perforation in bacillary dysentery but the local peritonitis may [illegible] adhesions which become a source of much pain and abdominal disco[illegible] many patients.

CHRONIC BACILLARY DYSENTERY:—In this condition, many of the ulcers do not heal but persist for a long time. They are usually superficial, vary in size and are commonly small and lenticular. The wall of the gut is very much thickened and undergoes a diffuse fibrosis. The sigmoid colon in such cases may be felt as a fairly hard tube. In very extreme cases, when the ulcerations are very extensive and there is considerable loss of the mucous membrane, the whole bowel looks like *a piece of leather.*

Epithelial hyperplasia with formation of polypoid overgrowths:—In less severe cases, some strands of intact epithelium always remain in between the cicatrices and the chronic ulcers. These may undergo much hyperplasia, leading to the formation of polypoid overgrowths. In some instances, small translucent cyst-like swellings may be seen in large numbers, due to the superficial granulation tissue blocking the mouths of the Liberkühn's follicles whose bases remain intact. The epithelial cells which undergo hyperplasia secrete a mucoid fluid which accumulates inside. A sort of retention cysts is thus formed. These may burst, discharging a jelly-like fluid which may contain dysentery bacilli in large numbers. Thus, they play an important part in the carrier problem.

Mesenteric lymph nodes:—These are usually enlarged but they never suppurate. Post mortem, dysentery organisms may occasionally be found in them, particularly in very severe cases.

Other lesions that may be encountered in connection with bacillary dysentery are the following:—

1. **Arthritis**—Synovial fluid is straw coloured and sterile on culture. It possesses considerable agglutinative power for the homologous organism.
2. **Neuritis**—It is found only in 1 per cent. of the cases.
3. **Ocular complications** are conjunctivitis and irido-cyclitis. The latter occurs only in Shiga infections.
4. **Pyæmic abscess of the liver**—This is due to invasion by secondary organisms from the portal tracts.

Note:—Arthritis, neuritis and ocular complications are all due to the effect of toxins.

Characters of the stool in acute bacillary dysentery

Naked eye appearances:—

1. Scanty but numerous.
2. Reaction—alkaline.
3. A characteristic alkaline odour is present.
4. Viscid, mucoid or mucopurulent flakes, intimately mixed with blood. Sometimes, the stool resembles blood-stained pus which is obtained after incising an abscess.
5. On keeping, a large amount of sero-sanguineous fluid may be found to separate out of the muco-purulent flakes which collect at the bottom.

In some cases, particularly in infection with E. dysenteriæ (Shiga), the stools may consist of pure blood with little mucus. In other cases, the stool may resemble a sero-sanguineous fluid, very much like meat-washings, and is passed frequently and in large quantities. These cases may be mistaken for cholera. When gangrene of the gut supervenes, the stool becomes very foul smelling and contains much greenish slough and dark coloured altered blood.

Microscopic appearances (Fig. 239):—

1. The exudate is very cellular.

2. Large numbers of pus cells and abundant mucus are present.

3. Many red cells which are discrete and normal in appearance are present; there is no clumping and rouleaux formation.

4. Many large refractile cells (macrophages) are present. They may show red cell phagocytosis and even slight movement and may be mistaken by the inexperienced observer as amœbæ. As to their origin, there is much difference of opinion. They are erroneously called "endothelial cells," but the latter are too highly specialised to show any phagocytosis. They may be derived from the wandering phagocytic cells, the so called "histiocytes," which always abound in an inflammatory condition of the bowels or of any other part of the body.

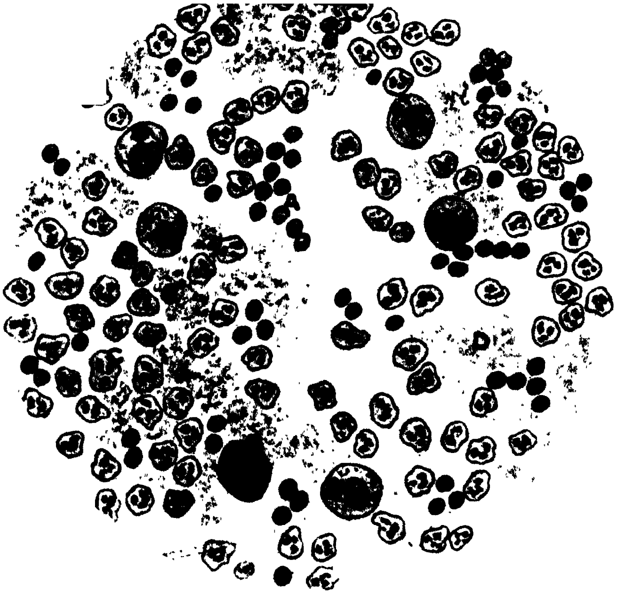


Fig. 239.

Photomicrograph of the stool from a case of acute bacillary dysentery.
A—Red blood corpuscles; B—Macrophages, some of which have phagocytosed red blood corpuscle; C—Polymorphonuclear leucocytes; D—Mucus.

5. In an acute case, in the earlier stages, there are comparatively few bacteria to be seen under the microscope, but as the disease becomes sub-acute or chronic, the bacterial flora increases remarkably. In the late stage, Entamœba coli and intestinal flagellates [illegible] appear.

Concomitants:—During the course of illness, the causative organisms become scanty and their place is taken up by other intestinal bacteria, such as Alcaligenes fæcalis, Salmonella morgani, Proteus vulgaris, Streptococcus fæcalis (Enterococcus) and other organisms resembling coliform bacilli.

Graham (1918) has studied the naked eye appearances and cytological changes in the stool of acute bacillary dysentery in its several stages. According to him, such pictures may give some information about the pathological changes occurring in the wall of the intestine, as shown in the following table:—

Stages of bacillary dysentery.	*Naked eye appearance.*	*Microscopical Characteristics (Cytological examination).*	*Pathological changes in the wall of the intestine.*
During first 24 hours	Clear mucus streaked with blood.	Numerous red and white cells which are well preserved. Epithelial cells are also found.	Intense congestion of the mucous membrane of the large intestine the glands of which secrete a large quantity of mucus.
During 48 to 72 hours	Evacuations consist of bright red blood, semi-opaque mucus and a clear fluid.	Large numbers of red cells, abundance of pus cells which may be clumped, epithelial and "macrophage" cells.	Intense congestion and even hæmorrhages. Surface of the mucous membrane covered with fibrin mostly marked over the rugæ. Wall of the gut thickened.
From the third to the fifth day ...	Stools consist of semi-opaque mucus with some blood and opaque fluid. Bright red blood is no longer a prominent feature.	Consist almost entirely of pus cells. A few red cells and macrophages.	Mucous membrane covered with a uniform layer of fibrin, may have sloughs. Gut wall congested and thickened.
About the fifth day	Little or no blood seen. Muco-purulent evacuation.	90 p.c. are pus cells. 10 p.c. consist of red cells (few only), Lymphocytes, endothelial, epithelial and plasma cells are also found.	Coagulative necrosis and ulceration of the mucosa are taking place.
After the fifth day ...	Fæcal matter reappears in the stool. Tags of mucus may be present.	Pus cells occur singly or in small groups of 3 or 4.	Stage of resolution.

In mild bacillary dysentery, gross evidence of blood disappears from the motion in a couple of days and purulent mucus is not seen. Semi-purulent mucus is found until the fifth day and at the end of a week, a fæcal stool may contain tags of mucus.

Carriers

It has been found that patients, recovering from an acute attack of bacillary dysentery, often excrete the organisms in their stool for a week or so. These are only *temporary carriers.* But there are others who continue to excrete the bacilli for more than three months and they are grouped as "*persistent carriers.*" The latter are the subjects of chronic dysentery with some unhealed chronic ulcers in their rectum where the bacilli may persist and continue to be discharged with little flakes of mucus. Or, large numbers of organisms may be present in the small "*pseudo-adenomata—the retention cysts*" which are often found in the mucosa. When the latter burst, the bacilli are discharged periodically with the fæces which thus becomes a source of infection. There is some difference clinically between Shiga and Flexner carriers. These are shown in the following table:—

Shiga carriers.	**Flexner carriers.**
1. All of them belong to persistent carriers.	1. The number of persistent carriers is very small.
2. Excretion of the bacilli is more constant.	2. Excretion of the bacilli is intermittent.
3. Relapses are frequent.	3. Relapses are not so frequent.
4. The subjects look ill and suffer from chronic dysentery and mental depression, i.e., they are invalids.	4. The subjects may enjoy apparently normal health.

THERAPEUTIC CONSIDERATIONS

Antiserum:—This is beneficial only in infection with E. dysenteriæ, Shiga which produces a powerful extracellular toxin. To be effective, the serum must be used as early as possible and in sufficient quantity so as to neutralise the absorbed toxin. As soon as a case of acute dysentery comes under treatment, arrangements for a rapid bacteriological diagnosis should be made, but, at the same time, antiserum administered. If the infection proves later on to be due to an organism other than E. dysenteriæ, Shiga, the specific serum therapy may be stopped.

In a case of moderate severity, the intramuscular route may be used. But if the infection is severe, the serum should be given intravenously, if possible. The method of administration, as recommended by *Graham,* consists of 60 to 80 c.c. of serum injected intravenously, followed by a transfusion of 200 to 300 c.c. or even more, of a 5 per cent. glucose in normal saline. This should be repeated every 6 to 8 hours if necessary, depending on the toxic symptoms manifested by the patient and the amount of fluid lost in evacuations. The risk of anaphylaxis should be borne in mind and it is always better to make a preliminary test for serum sensitiveness (*vide* page 191) before administering the whole serum, particularly by the intravenous route. An injection of atropine often minimises the untoward symptoms.

The antiserum is prepared commercially by immunising horses, first with killed and later with living organisms. It is available in the market, both as monovalent (Shiga) or polyvalent (containing both Shiga and Flexner) serum. The therapeutic effect of a polyvalent serum, however, depends on the amount and concentration of the Shiga antitoxin contained in it.

Bacteriophage:—Of late, treatment of acute and chronic dysenteries by means of bacteriophage has come into use almost as a routine. It is essential that the "phage" should be administered at the earliest opportunity before the mucous membrane of the gut is seriously damaged. To commence with, a mixed, polyvalent dysentery phage is used but wherever possible, it should later on be substituted by an "autophage" prepared from the patient's stool. The bacteriophage is usually supplied in ampoules of 2 to 3 c.c. and 3 or 4 such ampoules should be taken in 24 hours. It should be administered in an empty stomach, preferably with an alkali. No food of any kind should be allowed one hour before and after the phage is administered. In favourable cases, the number of stools rapidly diminishes, pain and tenesmus disappear and the stools become faeculent and free from blood.

Vaccines:—The use of vaccine in the treatment of acute bacillary dysentery has not been successful owing to the rapid course of the disease. But in chronic cases, it has some beneficial effect on the course of the illness. It should be remembered that a vaccine prepared from E. dysenteriæ, Shiga is very toxic and gives rise to a very severe local and general reaction. Hence, great care is necessary in its administration. Whenever possible the vaccine should be autogenous.

The most important use of the vaccine therapy is in the prophylaxis. This is carried out in the same way as in the case of the prophylactic inoculation against typhoid fever and cholera. Owing to the high grade of toxicity of E. dysenteriæ, Shiga, heavy doses cannot be given. To overcome this difficulty, *sero-vaccines* were introduced. The latter consist of heat-killed organisms which have been treated with sufficient specific antiserum to neutralise the toxic effects of the bacilli. In this way, large doses of the organisms can be administered without any untoward reaction. Usually two injections are given at an interval of a week or 10 days. The first dose consists of 0.25 c.c., containing 500 millions of Shiga organisms and 0.1 c.c. of serum, the second dose being double this strength. In practice, the prophylactic vaccine should contain both the important dysentery producing organisms, viz., E. dysenteriæ and E. paradysenteriæ in equal proportions. After the inoculations, sufficient agglutinins are developed in the blood within 3 weeks to protect the individual. In recent years, with the introduction of the *anatoxin* (formolised Shiga toxin) *by Ramon*, it is used for prophylactic purposes. This causes only a slight reaction but gives rise to the production of enough antitoxin in the blood to protect against an infection.

Table showing the differences between Shiga and Flexner groups of dysentery bacilli.

	Eberthella Dysenteriæ. *(B. dysenteriæ, Shiga.)*	**Eberthella Paradysenteriæ.** *(B. dysenteriæ, Flexner.)*
Distribution	Prevalent in the East.	Prevalent in the West.
***Biochemical*:—**		
Mannite	o	+
Indol	o	+
Toxins	Exotoxins and endotoxins.	Only endotoxin.
Serological	Homogeneous group.	Heterogeneous group.
Pathogenicity :—		
In animals (Rabbit) ...	+ + + (Very great).	+
In man	(i) Causes a much more acute disease.	(i) A very much milder disease.
	(ii) Tendency to hæmorrhagic stool more marked.	(ii) Tendency to hæmorrhagic stool less marked.
	(iii) Toxæmia more marked.	(iii) Toxæmia not so marked.
	(iv) Neuritis may occur.	(iv) Neuritis does not occur.
Carriers	Commoner. Usually invalid.	Less frequent. May enjoy perfect health.
Therapy	Antiserum is of distinct value.	Vaccine therapy, particularly an auto-vaccine, is beneficial. Serum therapy is of no value.

OTHER ALLIED DYSENTERY ORGANISMS

E. dysenteriæ, Schmitz (*B. ambiguus of Andrewes; Parashiga + of Dudgeon and Urquhart.*)

Culturally it resembles the Shiga's bacillus. Differential points are the following:—

(i) It forms indol.
(ii) It is less pathogenic for rabbits than Shiga bacillus.
(iii) It is inagglutinable with Shiga and Flexner sera.

E. dysenteriæ, Sonne (*Duval's bacillus; B. dispar of Andrewes*).

It resembles Flexner group of dysentery bacilli, but in type it resembles Escherichia coli more than the true dysentery bacilli. Differential points are the following:—

(i) Late lactose fermenter (usually in from 5 to 10 days).
(ii) Permanent acidity of milk with or without curdling.
(iii) Forming no indol.
(iv) Toxicity in animals is variable.
(v) Serologically a distinct race.
(vi) Cause of dysentery in Scandinavia.

Table showing sugar reactions of various dysentery bacilli.

Organisms.	Glucose.	Mannite.	Lactose.	Maltose.	Saccharose.	Dulcite.
E. dysenteriæ, Shiga ...	+					
E. paradysenteriæ, Flexner ...	+					
E. dysenteriæ, Schmitz	+					
E. dysenteriæ, Sonne ...	+					

+ = Acid and no gas; o = No change,
* Y strain of the Flexner group does not ferment maltose.

ISOLATION OF DYSENTERY BACILLI

Points to remember:—

1. Bacillary dysentery is essentially a local disease.
2. The bacilli remain confined to the large intestine and only in extreme cases may they be found in the mesenteric lymph nodes.
3. They do not invade the blood stream, i.e., there is no *bacteriæmia*.
4. Blood and urine cultures are always sterile.
5. Synovial fluids from cases of dysenteric arthritis are also sterile.

Isolation of the specific organism from fæces:—

Collection of material:—Samples of fæces are best collected in a *fæces-specimen tube* (*vide* Fig. 233 on p. 427), provided with a cork and carrying a metal spoon or scoop which fits into the tube. If the stool contains both fæcal matter and mucus, a portion of the latter should be included in the specimen.

The material most likely to yield a positive result is fresh stool, entirely unmixed with urine and the most suitable one is that obtained in the early stages, containing blood and mucus. Immediate examination is very essential, since the viability of dysentery bacilli is not very great.

It has been found that the addition of an equal volume of alkali (3 per cent. sodium hydroxide) to a dysenteric stool greatly facilitates the isolation of the dysentery bacilli (*Dudgeon* 1919).

If the specimen cannot be examined immediately, it may be kept in a condition suitable for culture by adding 2 parts of 30 per cent. solution of glycerin in 0·6 per cent. saline solution to 1 part of fæces (*Teague and Clurman*, 1916). Under such conditions, the dysentery bacillus can be recovered after being kept in the above solution for 6, 7 or 8 days.

In chronic cases, there is a great difficulty in isolating the specific bacilli from the fæces. The best results are obtained by passing a *sigmoidoscope* and taking a swab directly from the base of the ulcer.

Methods of examination:—After sampling, the material is inoculated in a MacConkey's plate or any other selective medium. After 24 hours' incubation at 37°C., the suspected colonies (colourless ones, i.e., non-lactose fermenters) are picked up and studied in the following ways:—

(i) Subcultures are made on agar slope and in broth.

(ii) Fermentation reactions are studied in glucose, mannite and lactose.

(iii) Morphological characters, particularly its motility, are studied from a hanging-drop preparation of the liquid culture.

(iv) The agglutination reaction of the isolated bacillus is tested with a high titre serum (stocked in a refrigerator). For carrying out the reaction, a saline emulsion of the colonies from agar slopes is mixed with one or two drops of the high titre serum on a microscopic slide and clumping or flocculation noted (*slide agglutination method*). *Dreyer's* macroscopic technique may also be used.

If the suspected bacillus has the following characters, it may be regarded as belonging to one of the classical types, i.e., Shiga or Flexner's group. Diagnostic criteria of the classical strains are as follows:—

1. *Non-lactose fermenters.*
2. *Acid and no gas in glucose.*
3. *Non-motile.*
4. *Mannite*—Fermented by Flexner's group; not fermented by Shiga's bacillus.
5. *Serological reaction*—Agglutinated by Shiga antisera = Shiga's bacillus.
   Not agglutinated by Shiga antisera but agglutinated by Flexner antisera = Flexner's group.

Note:—Sometimes the bacillus on first isolation is inagglutinable but rapidly becomes agglutinable by subculturing. If it is still non-agglutinable, it may belong to atypical dysentery bacilli and its biological characters should be thoroughly studied.

LABORATORY DIAGNOSIS

The diagnosis of bacillary dysentery involves the exclusion of amœbic dysentery and isolation of the specific organism from the fæces. Blood examination does not offer any great help in the diagnosis, as there is no bacteriæmia in this disease. Agglutination reaction may help to a certain extent, particularly in subacute and chronic stages and also in carriers.

A. Examination of fæces:—This includes the naked eye and microscopic examination of stool and isolation of the specific organism from the same. The latter has already been described (*vide supra*). For convenience, a chart is appended below to show the differential characters of the stool in amœbic and bacillary dysentery.

	Bacillary dysentery.	*Amœbic dysentery.*
Colour	Rose pink.	Brownish.
Odour	No foul smell.	Foul smell.
Character	Blood and mucus intimately mixed.	Altered blood mixed with fæces.
Reaction	Alkaline.	Acid.
Microscopical ...	Cellular exudates plenty.	Cellular exudate few.
	Polymorphonuclear leucocytes and macrophages plenty. Red cells are discrete. Bacteria few and usually non-motile. Entamœba histolytica absent. Charcot-leyden crystals absent.	Polymorphonuclear leucocytes few; macrophages nil. Red cells appear in clumps or rouleaux. Many motile bacteria present. Entamœba histolytica present. Charcot-leyden crystals present.

B. Examination of blood:—

1. *Blood count:*—There is no characteristic blood picture. As a rule, specially in the acute stage, there is leucocytosis with increase of the neutrophil polymorphonuclear variety. A secondary type of anæmia may be seen in chronic dysentery cases.

2. *Agglutination reaction:*—The patient's serum may be examined for agglutination reaction against known Shiga and Flexner's strain, as such reactions may be obtained from a patient suffering from bacillary and not from amœbic dysentery. The reaction is carried out exactly like the Widal's test in typhoid fever, with the exception that the mixture is kept in water bath at 55°C. for 4 hours.

Specific agglutinins begin to appear in the blood of patients suffering from acute bacillary dysentery from the second or third day of the disease and a positive result is usually obtained between the *fifth and twelfth day*. For Shiga infection, an agglutination reaction occurring in a dilution of 1 in 50 should be considered as a positive indication of the disease. Since normal serum may agglutinate Flexner's bacilli, no agglutination reaction should be considered diagnostic in a dilution lower than 1 in 250.

Note:—As already mentioned, the organism cannot be isolated from blood and hence, *blood cultures are of no use.*

THE SALMONELLA GROUP OF BACTERIA

The Salmonella organisms comprise a large group of closely related bacteria which are all pathogenic to men and animals. Although they have no action on lactose, they readily ferment glucose, usually with the production of acid and gas. They are widely distributed in nature, being often found in the intestines of man and animals and also as localised pyogenic infections in the latter. The common bacteria which are included in this group are the following:—

1 and 2. *Salmonella paratyphi* (B. paratyphosus A) and *Salmonella schottmülleri* (B. paratyphosus B). These are natural pathogens of man, causing enteric fever. They are not found as normal parasites in other animal species.

3. *Salmonella hirschfeldi* (B. paratyphosus C)—It is closely related to S. suipestifer (monophasic type) and is a natural pathogen of man, giving rise to enteric fever.

4. *Salmonella morgani* (B. morgan No. I)—It is often associated with summer diarrhœa of children and is also encountered as concomitants in inflammatory conditions of the large bowel.

5 and 6. *Salmonella aertrycke* (B. aertrycke) and *Salmonella enteritidis* (B. enteritidis Gaertner)- These are natural pathogens of rodents, causing enteric infections; the latter also causes an acute enteritis in cattle. In human beings, they cause an acute gastro-enteritis (acute bacterial food poisoning).

7. *Salmonella typhi murium* (B. typhi murium)—The causative organism of epidemic disease in mice. Accidental infection in human beings has been reported, causing acute gastro-enteritis.

8. *Salmonella suipestifer* (B suipestifer)—It was supposed to be the cause of "hog cholera" but now regarded as secondary invaders. The organism is also responsible for a small number of cases of food poisoning.

9. *Salmonella psittacosis* (B. psittacosis)—The causative organism of pneumonia in parrots Infections in human beings who are associated with sick parrots have been reported.

10 and 11. *Salmonella pullorum* (B. pullorum) and *Salmonella gallinarum* (B. gallinarum)- Both of these organisms are non-motile. The former causes "white diarrhœa" in old chickens and septicæmia in young chicks and the latter causes fowl typhoid.

MORPHOLOGY:—All the members of this group have an average dimension of about 2 to 3 μ in length and about 0·5 μ in breadth. They all possess peritrichate flagella and are motile. They are non-sporing.

STAINING REACTION:—They are stained readily with the usual stains and are all Gram-negative.

CULTURAL CHARACTERS:—All these bacilli grow very luxuriantly in the usual culture media, just like the Escherichia group. On *nutrient agar*, the growth is similar to that of E. coli, viz., moist, glistening, somewhat translucent heavy growth. In *broth*, there is a uniform turbidity. From the character of the growth, however, it is not possible to distinguish these organisms from one another or from the other members of the typhoid-coli-dysentery group of organisms. As they do not ferment lactose, they produce colourless colonies on MacConkey's plate.

BIOCHEMICAL REACTION:—

Sugar fermentations:—As already stated, these bacilli never ferment lactose or saccharose. But glucose, maltose and mannite are readily fermented with the production of *acid and gas*.

Litmus milk:—In the beginning (i.e., during the first 24 to 48 hours), there may be slight acidity which, however, changes to an alkaline reaction on keeping. There is never any coagulation of milk.

Gelatin is never liquefied and *no indol* (except S. morgani) is produced.

Formation of H_2S:—This is tested in the lead acetate agar medium (p. 74). Most of the members of this group can produce H_2S (*vide* table below).

Table showing the biochemical reactions of the important members of the Salmonella group.

Organisms.	Lactose.	Glucose.	Litmus milk.	Lead acetate agar.	Indol.
S. paratyphi ...	o	AG	A	–	
S. schottmülleri ...	o	AG	A-Alk.	+	
S. hirschfeldi ...	o	AG	A-Alk.	+	
S. enteritidis ...	o	AG	A-Alk.	+	
S. ærtrycke ...	o	AG	A-Alk.	+	
S. suipestifer ...	o	AG	A-Alk.	+	
S. typhi murium	o	AG	A-Alk.	+	
S. morgani ...	o	AG	A-Alk.	+	

A—acid; AG—acid and gas; Alk.—alkaline; A-Alk.—Initial acidity followed by alkalinity.

The sign + in lead acetate agar signifies blackening of the medium due to the formation of H_2S; the sign - indicates no change.

S. morgani—Excepting glucose, it does not ferment any other sugar. It has been found to be a frequent cause of summer diarrhœa in children in England, although it is not the only one for that condition. Other organisms found to be associated with the disease are—Proteus vulgaris, Eberthella paradysenteriæ, Eberthella dysenteriæ Sonne, Alcaligenes fæcalis, etc.

VIABILITY:—There is no doubt that the Salmonella group of organisms is more resistant than either the Eberthella or the Escherichia group. In the stool, the paratyphoid bacilli have been found to remain alive for many days, even if they are exposed to open air. They can also stand a considerable amount of desiccation without losing their viability. In water, the Salmonella organisms may persist for months without any loss of vitality. They cannot, however, resist the action of gastric juice for more than a couple of minutes. Moist heat of 60°C. kills all the bacteria in a few minutes. It has been found that about 7 to 8 per cent. of common salt inhibits the growth of Salmonella bacilli. This is important to remember as these bacilli are sometimes found as contaminating organisms in tinned meat and fish.

TOXIN PRODUCTION:—These bacilli do not produce any extracellular toxin. The poisonous product is contained in the substance of the bacterial body which liberates it only when the latter is disintegrated or dissolved. This endotoxin is thermostable and can resist heating to 100°C. for half an hour without undergoing decomposition. As it is considerably heat-resistant, it can withstand cooking processes specially when the latter are imperfectly undertaken. When injected intraperitoneally or intravenously, this toxin produces fatal results in laboratory animals.

SEROLOGICAL REACTIONS:—When any member of the Salmonella group is injected into an animal, either in its peritoneal cavity or in the vein, there are produced both specific and group agglutinins in the blood of the injected animal. These are used for diagnostic purposes in the same way as in the case of typhoid fever. Besides these, there are produced precipitins and complement fixing bodies in the serum of the inoculated animal or in that of a person who had suffered from an infection.

Like all other flagellated bacteria, the Salmonella group of organisms possesses two types of antigens—H or flagellar (heat-labile) and O or somatic (heat-stable)—both of which give rise to their corresponding agglutinins. Andrewes (1922) has further shown that *H antigens* of certain strains in this group are *diphasic in nature*—one phase being specific for the particular serological type and the other or group phase being common to related organisms. In the *type phase*, the strain can be identified by direct agglutination, while in its *group phase*, it is indistinguishable by direct agglutination and can only be differentiated with certainty by cross agglutination or absorption of agglutinins. In this way, the antigenic nature of the various strains has been found to be as follows:—

(i) *S. paratyphi* (B. paratyphosus A) and *S. enteritidis* (B. enteritidis Gærtner) are both monophasic and therefore can be identified by direct agglutination.

(ii) *S. schottmülleri* (B. paratyphosus B), *S. ærtrycke* (B. ærtrycke) and *S. suipestifer* (B. suipestifer) are all diphasic. In their group phase, they can only be identified from one another with certainty by absorption of agglutinins, while in their type phase, they possess an antigenic factor peculiar to themselves, rendering an easy separation by direct agglutination.

PATHOGENICITY

It has already been pointed out that the members of this group are very widely distributed in nature, both in animals and human beings, and the bacilli may be scattered from such sources contaminating foodstuffs, beverages, milk, fruits and water.

Salmonella infection in animals

There is a wide range of animals which may suffer from various kinds of Salmonella infections. The most important of them are the rats, mice, pigs, sheep, cattle and birds. Such infection is characterised by the production of a septicæmia with localised lesions in various parts of the body, such as arthritis, enteritis, metritis, mastitis, osteomyelitis and pyæmic abscesses. Large numbers of bacilli are present in these lesions, as well as in the intestines of such animals which may become a source of danger to human beings, either through the infected flesh or through bacilli which are excreted from the intestines and which contaminate otherwise healthy foodstuffs.

Salmonella infection in man

Modes of infection:—

(1) By eating meat of an animal actually suffering from Salmonella infection. The meat is usually imperfectly cooked.

(2) By taking foodstuffs and drinks contaminated with the injurious germs excreted from the intestines of man and animals.

(3) From human carriers who may harbour the bacilli in their intestines and who may periodically or continuously discharge them in their stool. In this case, like typhoid infection, *finger, food* and *flies* always play very important rôles.

It should be remembered, as already pointed out, that the members of this group are somewhat resistant to unfavourable circumstances, such as heating, desiccation and unsuitable environmental changes, and it is for this reason that imperfectly cooked and made-up meat is often a fruitful source of infection. Contaminated milk or drinking water is sometimes responsible for an outbreak of localised epidemics.

The bacilli which are taken in along with the food and drink may be killed by the gastric juice, but if they can pass intact through the stomach into the alkaline duodenal content, they multiply very rapidly and may either get into the blood stream producing bacillæmia, or produce an intense irritation throughout the gastro-intestinal tracts with varying grades of intoxication. According to the nature of then effects, Salmonella organisms may produce the following lesions in man:—

(A) *Salmonella fevers* with generalised infection running an acute or subacute course, resembling very much the course of a typhoid fever.

(B) *Salmonella gastro-enteritis* with intense irritation of the gastro-intestinal tracts (Bacterial food poisoning).

(A) Salmonella Fevers

The organisms which are chiefly responsible for these fevers are the paratyphoid group, viz., the S. paratyphi (B. paratyphosus A), S. schottmülleri (B. paratyphosus B) and S. hirschfeldi (B. paratyphosus C).

Incidence:—It should be noted that in our country the *S. paratyphi* (B. paratyphosus A) *infection* is very much more common than the others. As the typhoid and the paratyphoid groups of organisms are very closely related and their mode of infection and the pathogenic lesions are somewhat similar, both the infections may co-exist in the same person.

Pathogenesis:—As stated above, the organisms usually gain entrance into the human system through contaminated food and drink. If they can pass unharmed through the stomach into the duodenum, they rapidly multiply and pass from the alimentary canal into the mesenteric lymph nodes and then into the general blood stream giving rise to, as in typhoid fever, a condition of bacteriæmia. The lesions produced in this infection are, however, never so pronounced as in the case of the typhoid infection. For instance, although the lymphatic elements of the intestines and other parts of the body may show hyperplasia, ulceration of the Peyer's patches is never so marked and the serious consequences, such as perforation and peritonitis, which are so much dreaded in typhoid fever, are hardly, if ever, present. Although the general course and the symptomatology of paratyphoid infection are like those of a mild case of typhoid fever, gastro-intestinal manifestations, such as nausea, vomiting, diarrhœa and colitis, may be well marked in some cases, specially in S. schottmülleri and S. hirschfeldi infections. During the course of the infection, as well as in the carrier state, large numbers of bacilli are excreted in the fæces. In the case of a carrier, like the typhoid infection, the gall bladder forms the most important reservoir for the organisms, but urinary and pure intestinal carriers are also known to occur. The blood changes and the development of agglutinins are exactly on the same line as in the typhoid infection.

Some members of the Salmonella group occasionally produce *fevers of short duration*, associated with marked gastro-intestinal disturbances. These are described under acute bacterial food poisoning. Besides the above, *fevers of considerable duration* may be produced by infection of the urinary tracts. There are minor abdominal symptoms preceding the attack which consists of a febrile paroxysm with chill and rigor. The fever may be either continuous or remittent in type. The *pathological condition* is usually limited to the *urinary tracts*, particularly the pelvis and calyces of the kidneys and also, to some extent, the ureter and the bladder. A true pyelitis or pyelonephritis is produced. The urine in such cases is turbid, containing pus, albumen and almost a pure culture of motile Gram-negative bacilli. These cases are usually passed clinically as "*B. coli pyelitis*" and the true nature of the infection is not determined until full bacteriological examination of the urine is carried out. The two most important organisms which are found in such conditions are *S. paratyphi* (B. parratyphosus A) and *S. morgani*. It is a common experience of many pathologists to find non-lactose fermenting Gram-negative motile bacteria in the urine sent for examination for "*B. coli*" *infection* and on detailed examination, such organisms may be found to belong to one or other member of the Salmonella group.

(B) Salmonella Gastro-enteritis

(Acute bacterial food poisoning)

This condition was previously considered to be due to "ptomaine poisoning." It was thought that these ptomaines were toxic aminobodies formed from animal proteins when the latter were in an advanced stage of decomposition. It naturally follows, therefore, that the poisoning effects should occur only when decomposed meat or fish are eaten by men in sufficiently large quantities. This idea prevailed for a long period until the nature of the disease was questioned, when it was found that people suffered from this condition on eating apparently healthy-looking meat, fish and vegetable food stuffs. Besides, it was also found that whereas ptomaines in reasonable amounts were non-toxic to laboratory animals, the suspected meat, fish or other food stuffs were highly toxic to these animals when given by the mouth. From these observations, the idea gradually gained ground that the so-called "ptomaine poisoning" was not due to ptomaines but to some other agents. Thus, the etiology of the sickness gradually passed from chemical poisoning to microbial infection.

Acute food poisoning, as we understand it in these days, is an illness caused by eating food stuffs infected with some member of the Salmonella group and is characterised by a severe gastro-enteritis of sudden onset, with varying grades of fever and toxæmia.

Description of a typical case:—A person, having eaten some infected meat, fish or imperfectly cooked, prepared food or tinned foodstuff, goes to bed at night, but in the morning is attacked with a severe headache, abdominal pain, nausea, wretching, vomiting and diarrhœa, with the passage of mucus and sometimes blood in the stool. Fever of some degree is always present. The general discomfort and restlessness become very great and the patient feels miserable. There may be intense thirst, cramps, and in a virulent infection, coma, followed by death. In moderately virulent cases, the illness lasts for a period varying from 4 to 7 days, after which the symptoms abate and the condition of the patient improves.

Bacteriology:—The common bacteria which have been found to cause acute food poisoning are, in order of their frequency, as follows:—

(i) S. enteritidis (B. enteritidis of Gærtner).
(ii) S. ærtrycke (B. ærtrycke)—Aertrycke is the name of a Belgian village where an outbreak of food poisoning occurred and where the organisms were isolated.
(iii) S. suipestifer (B. suipestifer).
(iv) S. morgani (B. morgan No. 1).
(v) S. hirschfeldi and S. schottmülleri (B para. C and B).
(vi) A large group of closely allied organisms.

Of the above mentioned organisms, the two which are most commonly responsible are the *S. enteritidis* and *S. ærtrycke*. It should be remembered ***that when the disease occurs by eating the flesh of an already diseased animal, S. enteritidis is the commonest infecting agent, whereas if the original meat is innocent but it becomes contaminated during the process of preparation, S. ærtrycke should be suspected.***

Sources of infection:—About 60 to 70 per cent. of the outbreaks of food poisoning are due to infected meat from oxen and pigs. Mutton and chicken are rarely incriminated. In our country, goats are hardly, if ever, infected. Ducks may be involved but wild games are free from infection. Amongst fish, the commonest one is the shrimp. In our country, some very dangerous varieties of fish in this respect are the mango-fish and the hilsa-fish which are prevalent during the hot seasons and which are liable to get the infection from human excreta in fresh water streams passing through city areas. For instance, these varieties of fish are very plentiful in the river Hooghly, where faecal pollution is very considerable.

Types of food:—Meat, fish and milk are most important but vegetables, cereals and very occasionally, fruits may be responsible. The danger is specially marked when the above articles of diet are either imperfectly cooked and then kept for some time before being consumed or made up into various kinds of prepared foods, the process involving imperfect cooking and liability to contamination. The following are some of the examples:—

Meat, sausages, pâtés, chops, pies, cutlets and similar stuffed foods, pickled ham, etc.

Fish, tinned fish, pickled fish, smoked fish, fish imperfectly fried with a coating of some other material over it.

Milk, ice-creams, pastries, puddings and other milk products. Stale milk is also important.

The following table may give an idea about the nature of the foodstuffs which were incriminated in various epidemics in Great Britain.

Nature of food.		*Per cent.*
Canned meat, canned marine products and canned fruits	...	30·5
Milk and milk products	...	14·8
Made-up meat	...	26·6
Manipulated meat	...	4·9
Fresh meat	...	16·3
Fruits and vegetables (including salads)	...	3·9
Other foods	...	2·9

Seasonal prevalence:—Most of the outbreaks occur during the warmer months, the hot weather favouring the rapid growth and multiplication of the infective organisms. In our country, seasonal increase is also due to the variety of the food material available during any particular season. For instance, mango-fish and hilsa-fish which are sometimes incriminated are available only during the warm humid months.

Modes of infection:—

(a) Meat used for consumption may be obtained from an animal which is infected during its life time and may have been actually suffering from the disease, when slaughtered.

(b) The animal may be quite healthy and innocent, but its meat is contaminated after slaughter, during the course of dressing or preparation for eating. It should be definitely remembered, however, that both the prepared food as well as the flesh itself, may appear quite normal to the ordinary inspection, although very heavily contaminated with an infecting micro-organism. Normal good meat may be contaminated in the following ways:—

(i) In the slaughter house where infected animals may be brought in by dealers to be slaughtered.

(ii) By the fæces and fomites of carriers and also by infected flies.

(iii) From rats and mice which often suffer from Salmonella infection, and therefore, harbour the bacilli in their intestines. The danger from the rats is very considerable, particularly in our country where people do not use meat safes or other protectors for prepared foods, to any great extent.

THERAPEUTIC CONSIDERATIONS

Although the injection of these group of organisms into animals gives rise to the production of agglutinins, precipitins and complement fixing bodies in their blood, the therapeutic use of *immune sera* in actual cases is not attended with any good result. On the other hand, the use of *vaccines* is often beneficial, both for prophylaxis (S. paratyphi and S. schottmülleri) as well as for curative purposes (subacute or chronic Salmonella infection of the urinary tracts and intestines). It should, however, be remembered that such vaccine therapy cannot be of any use to an acute bacterial food poisoning, a condition which is sudden in onset and which lasts only for a short period.

Prophylaxis:—

(1) Fresh and well cooked meat, consumed immediately after cooking, is never followed by food poisoning.

(2) Made-up and prepared foods should be avoided. Sausages, pâtés, meat pies, ice creams and similar preparations are potentially dangerous.

(3) Milk should not be kept a long time after being boiled.

(4) In our country, shrimps, mango-fish and hilsa-fish should not be taken unless quite fresh and thoroughly cooked.

LABORATORY METHODS OF DIAGNOSIS

(A) ***Of Salmonella fevers:***—These may be considered exactly like E. typhi (B. typhosus) infection, i.e., blood culture, stool culture and agglutination reaction.

In doing the Widal's test in a case of paratyphoid infection, it should be remembered that in Salmonella paratyphi (B. paratyphosus A) infection, a positive agglutination reaction in a dilution of 1 in 25 of the serum may be considered as the minimum standard for diagnosis. In the case of Salmonella schottmülleri (B. paratyphosus B), however, the lowest diagnostic titre of the serum usually accepted as positive is a dilution of 1 in 50. Such a diagnostic criterion refers only to persons unvaccinated against these organisms. For the inoculated persons the same procedure as in the case of typhoid fever should be adopted.

(B) ***Of Salmonella gastro-enteritis*** (acute bacterial food poisoning): —For the proper investigation of an outbreak of food poisoning, one should proceed carefully and systematically, always keeping in mind that it is the contaminated food material which is primarily responsible. Therefore, it is necessary, for the purpose of an investigation, to try to find out and secure a specimen of such **foodstuff** which appears to be the vehicle of infection. This should be subjected to a bacteriological analysis. A portion of the meat, food or a little of the contaminated material is either mixed with saline or put into nutrient broth and incubated for 8 to 12 hours. A loopful of the saline emulsion or the broth culture is then spread over a couple of plates containing one of the special media. For ordinary purposes, MacConkey's neutral-red bile salt lactose agar is quite useful. After necessary incubation of the inoculated plates, the Salmonella organisms will appear as colourless colonies (non-lactose fermenters) which may then be subjected to other studies, viz, motility, staining reactions, biochemical characters and agglutination tests.

To establish a bacteriological diagnosis of **a case** of food poisoning, the *vomited material* and the *stool* of a patient actually suffering from the disease should be examined for the presence of the causative organisms. This may be done exactly on the same lines as in the case of the typhoid and paratyphoid infections, i.e., the cultures are first made, which are then subjected to biochemical and serological tests.

In the case of a suffering patient, the *blood* should always be examined. A general survey of the leucocytes should be made and the presence of malarial parasites looked for, because *in the tropics,* **pernicious type of malaria** *may produce symptoms exactly similar to those of acute bacterial food poisoning.* There is always an increase in the number of the leucocytes and the percentage of the polymorphonuclear (neutrophil) variety is raised. But the most important point about the blood examination is the demonstration of the agglutinins for S. enteritidis and S. ærtrycke. An infected person will always give a positive agglutination reaction within 4 to 5 days, after which the titre limit rapidly rises up to the third week.

During the laboratory investigation of a case of acute bacterial food poisoning in our tropical countries, the possibility of **cholera asiatica** *should be borne in mind,* as the two conditions may occur simultaneously in a locality. Apart from the usual procedure for the isolation of the Salmonella group of organisms, a bacteriological examination for Vibrio comma (V. choleræ) should be undertaken. Examination of the specific gravity of the blood is also very useful in such occasions.

In holding a **post mortem examination** on a fatal case of Salmonella food poisoning, heart's blood, spleen pulp, intestinal content and bile should be collected for a thorough bacteriological diagnosis.

In the investigation of the **carrier problem,** the fæces should be examined for the offending organisms and the blood serum for the presence of agglutinins.

Note :—To prove the serological identity of the isolated organism in the Salmonella group, both direct agglutination and agglutinin absorption may be necessary. For monophasic types, a direct agglutination will suffice, whereas for diphasic types, the technique for the absorption of agglutinin is essential before the strain can be recognised with certainty.

ESCHERICHIA COLI

(Bacterium coli; Bacillus coli communis)

HISTORICAL:—It was first described by Escherich in 1885 in connection with his studies on the bacterial content of the stool of new-born babies. He found that this organism first appeared in the fæces as soon as babies began to suckle.

HABITAT:—E. coli is very ubiquitous and is widely distributed in nature. It is always the principal organism of the bacterial flora of the fæces of man and all other animals. Consequently, it is found wherever there is pollution with their excreta. Thus, it is present in the soil, water, sewage, vegetables, etc. It and some of its allied strains are also found in grasses, grains and decomposing vegetable matter, even when there is no fæcal pollution. It appears that some strains of E. coli and its variants may live a saprophytic existence outside the animal body.

MORPHOLOGY:—It is a rod-shaped bacillus of about 2 μ in length. It possesses 4 to 8 *flagella*, arranged in a *peritrichate* manner and is therefore *motile*. But its motility is never so active and vigorous as that of Eberthella typhi. It has *no capsule* and *does not form any spores*.

STAINING REACTIONS:—It stains readily with ordinary aniline dyes and is Gram-negative.

CULTURAL CHARACTERS:—

Agar—It grows readily in ordinary culture media producing luxuriant, moist, somewhat bluish and semi-opaque colonies which may overlap if the inoculum is heavy. The surface of the colonies is smooth and glistening. Sometimes, there is much accumulation of gas inside the medium.

Broth—It produces uniform turbidity with a heavy growth and an unpleasant fœtid smell.

Special media. It produces pink colonies in MacConkey's, Conradi's and Endo's media. In brom-cresol purple medium, the colonies are yellow.

BIOCHEMICAL REACTIONS:—It is aerobic and facultatively anaerobic.

Fermentation reactions:—It ferments all the sugars with the production of acid and gas The organism does not form indol, liquefy gelatin nor does it form H_2S.

Voges-Proskauer reaction is negative (for the technique vide p. 89).

Methyl-red test is positive (for the technique vide p. 89).

Litmus milk—It produces acid with coagulation.

Hæmolysin production—Certain pathogenic strains of E. coli, isolated from cases of urinary tract infection, produce powerful hæmolysins.

Formation of reductase—The organism has the power to reduce various dyes (methylene blue) and nitrates (for the technique vide p. 88).

Koser's test (p. 89)—When Escherichia coli (of fæcal origin). is allowed to grow in Koser's medium, no growth is detected as the organism is unable to utilise the citrate.

VIABILITY:—*Escherichia coli* is readily killed when exposed to a temperature of 60°C. for 10 minutes, but it can retain its vitality and survive even for weeks and months when protected from direct sunlight. It also resists desiccation for a much longer period than its more pathogenic allies. In water, it retains its vitality for a prolonged period. In contaminated soil with plenty of moisture, E. coli can live indefinitely.

In this connection, the action of certain selective substances on the growth of E. coli may be considered. Of the two most important elements, salts of bile exert an inhibitory effect on other bacteria but favour the growth of the typhoid-coli group; whilst brilliant green, in a dilution of 1 in 150,000, inhibits the growth of E. coli but greatly favours the cultivation of Eberthella typhi. Other basic dyes, such as crystal violet, gentian violet, etc., while inhibiting the growth of the Gram-positive bacteria, do not materially influence that of E. coli. Hence, these dye materials are extensively used for the preparation of various kinds of special selective culture media.

TOXIN PRODUCTION:—No exotoxins are produced. The poisonous products appear to remain inside the substance of the bacterial body and are liberated only on the lysis of the organisms. Certain strains, producing pathogenic lesions, specially in the urinary tract, produce a powerful hæmolysin.

SEROLOGICAL REACTIONS:—The injection of gradually increasing doses of the organisms gives rise to the production of specific agglutinins, precipitins and other bacteriolytic substances. A normal individual may show the presence of some amount of agglutinin in his serum, varying from 1 in 10 to 1 in 20, owing perhaps to the constant presence of the bacilli inside his body. Although agglutinins are produced by such inoculation, it is not possible to utilise the production of agglutinins in the matter of diagnosis, owing to the fact that strains of E. coli vary very widely regarding their serological reactions. Just to express it more simply, a strain may resemble E. coli typically in morphology, fermentative and other biochemical reactions but still differ widely from the same in its serological reactions. Hence, serum reactions, unlike in typhoid fever, are useless for diagnostic purposes. Only the hæmolytic strains, isolated from urinary tract infections, form a special group and their serological reactions are found to be fairly constant and reliable.

ANIMAL INOCULATION:—When injected into the subcutaneous tissues of an animal, E. coli produces a local inflammation with an abscess formation, but no generalised infection takes place. When injected intravenously or intraperitoneally into a rabbit or guinea-pig in sufficient doses, it causes death of the animal in a day or two, with collapse and symptoms of diarrhœa.

PATHOGENICITY

It has been said that E. coli (B. coli communis) is necessary in life and is very helpful to all animals and men to complete the digestive process in the alimentary tract. Besides this digestive [illegible]tion, it has another very important rôle in the economy of animal [illegible] it offers a certain amount of protection against pathogenic micro-org[illegible] by rapidly over-growing the latter. An example of this action has [illegible] been seen in the case of E. typhi (B. typhosus). It is quite justifie[illegible]ore, to suppose that there is *a sort of symbiosis between it and the animal being which adapts itself to the action of this particular bacterium and an equilibrium is maintained under* ordinary conditions of life. But whenever this balance is upset, the organism gets a chance to go beyond its legitimate site and invade distant tissues and organs producing pathological effects. The conditions which bring about this abnormal state are convalescence after prolonged illnesses, exhaustive influences, trauma, infective processes in the portal tracts and local loss of resistance, either by disease or by environmental changes, such as constipation, inflammatory changes in the neighbourhood of the gut, bands, kinks, twists, etc. of the intestines.

Escherichia coli (B. coli communis) is a pyogenic organism and its pathogenic lesions are, therefore, associated with pus formation. But as it does not produce any powerful toxins, there are no marked signs and symptoms of general intoxication. One of the important features is the production of pyrexia without its corresponding toxæmia. The ***pathogenic lesions*** of Escherichia coli in man may be considered as follows:—

Blood:—E. coli, under exceptional circumstances, may enter the circulation and cause a condition of bacteriæmia and septicæmia. On such occasions, there is more or less a remittent type of temperature, associated with chill, rigor and sweating, and the infection follows a course resembling that of an infection by the enteric group of organisms. Usually it is not fatal, provided it is not complicated by abscess formation in the internal organs.

Liver and Gall bladder:—E. coli is a very important agent in the production of cholecystitis, cholangitis and multiple foci of suppuration in the liver. The bacilli may reach the liver and gall bladder from any infective pyelephlebitis in the portal area, such as suppurative appendicitis or infected and thrombosed piles. From such a source of infection, a septic thrombus may be dislodged and carried over by the circulating blood to the liver. In cases of biliary calculi lodged in one of the ducts and producing an incomplete obstruction to the passage of bile, the organisms often get into the gall bladder producing a suppurative cholecystitis. Further spread of infection through the bile ducts into the liver substance produces suppurative cholangitis with multiple abscess formation. It may be noted that the above pathological conditions are very serious and often fatal. They are associated with long continued pyrexia with chill, rigor and sweating, an enlarged, painful and tender liver and jaundice.

Portal tracts:—Any inflammatory condition in this area, such as ulcerative colitis, peritonitis, appendicitis and suppurative piles, is associated with the presence of E. coli. All ulcerative conditions of the large intestine are secondarily invaded by the colon group of organisms which help to maintain the conditions and make them chronic. In weak and debilitated persons and also in terminal conditions, E. coli may start a primary colitis, producing all the signs and symptoms of dysentery. In perforative and other forms of peritonitis, dependent on surgical diseases of the gastro-intestinal tracts, E. coli is always present along with Streptococcus fæcalis and other anærobic spore-bearing bacteria. The two very important diseases which should always be remembered in this connection are *appendicitis* and *inflamed hæmorrhoids*.

In both these conditions, there is always a potential danger of infective pyelephlebitis of the corresponding veins (Fig. 240). As already discussed, such a condition may be followed by very grave consequences.

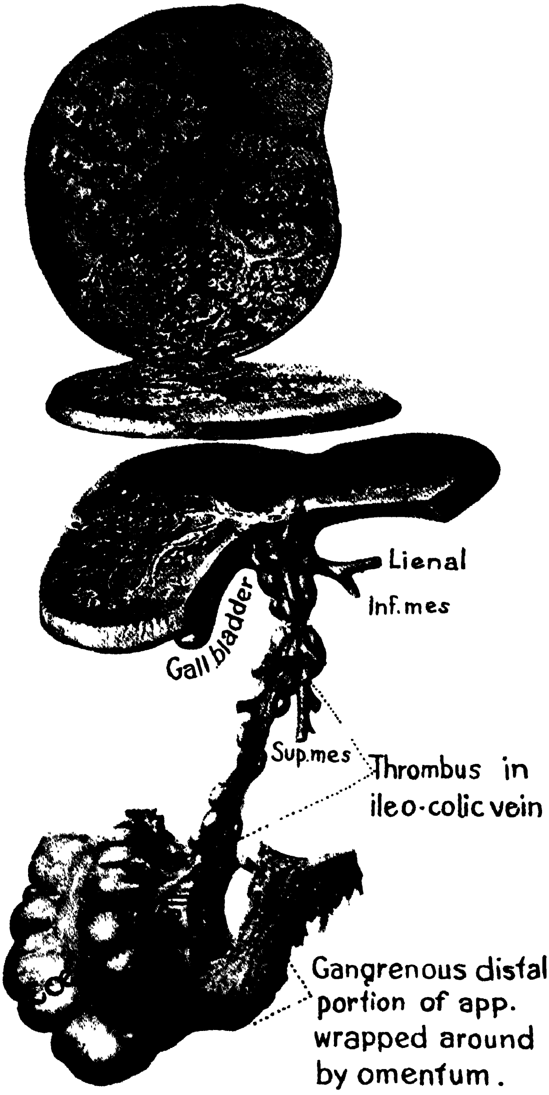

Lienal
Inf. mes
Gall bladder
Sup. mes
Thrombus in ileo-colic vein
Gangrenous distal portion of app. wrapped around by omentum.

Fig. 240.

Suppurative thrombo-phlebitis of the mesenteric veins with multiple abscesses in the liver, following appendicitis. The picture illustrates beautifully the condition of the veins of the portal system and of the liver, as found at autopsy.

(*From Kelly and Hurdon's "the vermiform appendix and its diseases"; by kind permission of W. B. Saunders and Company.*)

Genito-urinary tract infection:— These infections consist of *pyelitis, pyelonephritis, abscess of the kidney, cystitis* and *ascending infections*. The genito-urinary tract is a very favourite site for infection by Escherichia coli. The condition is more common in females than in males. The areas which are commonly affected are the pelvis of the kidney and the urinary bladder.

In the *kidney*, the bacilli most commonly cause a catarrhal inflammation of the mucous membrane of the renal pelvis, calyces and the adjacent portion of the ureter (*pyelitis*). There is congestion, swelling and desquamation of the epithelial cells, together with a copious exudation of leucocytes, red cells and serum. There is usually some obstruction to the free flow of pus and urine down the ureter owing to the swelling of the wall of the latter and the consequent diminution of its lumen. It is for this reason that there is some degree of dilatation of the pelvis and calyces and the fluid inside them is always under a certain amount of tension. The process may stop there and the entire inflammation may subside and the mucous membrane restored to its normal condition. But in unfavourable cases and with infection by virulent strains, the process may spread from the papillæ to the substance of the kidney, producing multiple foci of suppuration (Figs. 241 and 242). This condition is known as *pyelonephritis*.

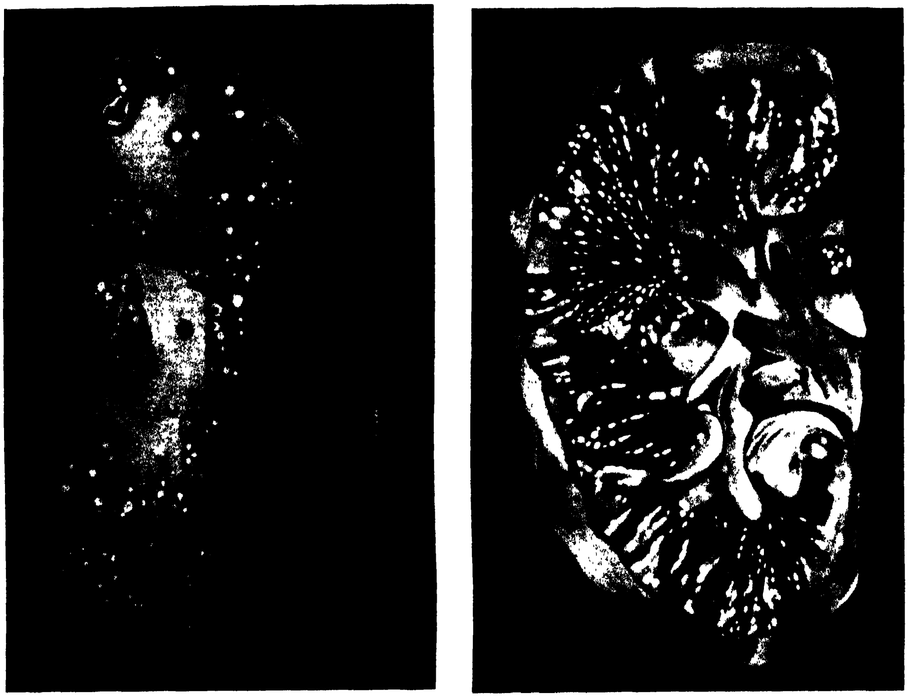


Fig. 241. **Fig. 242.**

Diffuse suppurative nephritis. The white spots represent areas of pus formation

This is a very serious pathological condition, producing grave complications, such as failure of the kidney functions with uræmia, extension to the perinephric tissues, producing perinephric abscess and general blood infection.

The *urinary bladder* is often the seat of inflammation produced by E. coli. This (cystitis) may result either secondarily to an infection of the pelvis of the kidney or as a primary condition. Obstruction in the urinary passages, such as an enlarged prostate, stricture of the urethra and calculus in the bladder, often markedly predispose to such an infection. A very serious complication which may originate under this condition is the spread of the infection upwards, either along the lumen of the ureters or the periureteral lymphatics or possibly both, producing what is known as an *ascending pyelonephritis* with all its concomitant dangers. It is a much dreaded complication to surgeons owing to its risks.

Escherichia coli infection in the females:—In the female sex, E. coli infection of the urinary tract is very frequent and should always be kept in mind whenever one has to deal with intermittent pyrexia in women. The reasons for this increased incidence have been said to be due to the shortness of the urethra, strain and pressure effects during pregnancy, loaded sigmoid colon in constipation and trauma during sexual intercourse. *Pregnancy* and *puerperium* are special predisposing factors. The enlarged gravid uterus exerts a marked pressure effect on the ureters, causing difficulty in the free flow of urine and producing some loss of resistance to infection of the pelvic and ureteral mucous membranes.

Clinical picture of a case of E. coli infection of the urinary tract consists of an intermittent pyrexia with chill and rigor, followed often by profuse perspiration and unassociated with a corresponding amount of toxæmia. There is pain and difficulty in micturition and passage of a strongly acid, turbid urine with a fishy odour. Occasionally, the pain in the loin may be so severe as to simulate renal colic and there may be passage of blood with urine. Along with the urinary complaints, there are often some signs and symptoms of colitis, such as pain and tenderness along the course of the colon, diarrhœa with mucus and sometimes blood in the stool, flatulence and indigestion.

Characters of the urine in E. coli infection of the urinary tract:—

Physical appearances.—It is opalescent or turbid, with copious sediment of pus. It has a strong, fishy odour and is markedly acid in reaction. It may or may not contain blood.

Chemical features.—Such an urine always contains varying amounts of albumen and gives reactions for pus and blood.

Microscopic appearance of the sediments—Large numbers of pus cells, varying numbers of red blood cells, specially in the acute stage and many motile Gram-negative bacilli are seen.

Cultural characters:—When a sample is cultured in suitable media, there is a vigorous growth of E. coli. Sometimes, the nutrient agar is pushed and broken up, due to the formation of much gas inside the medium by some of the strains.

THERAPEUTIC CONSIDERATIONS

Vaccines:—E. coli infections are sometimes treated with vaccines, often with very beneficial results. The cases suitable for vaccine therapy are the urinary tract infections, cholecystitis and sometimes ulcerative colitis. It is always desirable to use an autogenous vaccine and to start treatment after the acute stage of the inflammatory process has passed into a subacute one. It should always be started with a small dose and 5 millions of the organisms are often enough for a case of pyelitis or cystitis. Of course, the vaccine therapy should be combined with all recognised accessory forms of treatment. As already stated, the hæmolytic strains of E. coli invading the urinary tracts belong to one and the same serological group and for this reason, stock vaccines administered in such an infection will do good. But in all non-hæmolytic infections, autogenous vaccines should be used.

Apart from this specific therapeutic administration of E. coli vaccine, there is another use. This is in connection with the non-specific protein therapy for the amelioration and often cure of chronic inflammatory processes in the joints, tendons, pelvic organs in the females and other places in the body. For this purpose, a big dose of the vaccine may be used, often 100 to 500 millions, and injected intravenously to produce a protein shock. A severe reaction with chill, rigor and high fever sets in, lasting for varying periods. But with the subsidence of the reaction, the other pathological conditions begin to improve. The injections may be repeated in increasing doses at intervals of about 7 days. It may also be used as an ordinary vaccine but in comparatively bigger doses, for the treatment of *rheumatoid conditions*, when the intestinal tracts are suspected to be the source of septic absorption.

Bacteriophage:—A very potent "phage" is always present in the intestinal canal of all animals and in detectable amounts in other places where E. coli gains entrance. Thus, it is found in sewage and contaminated water supply. Unfortunately, the therapeutic value of this " phage " is very limited, although its presence is responsible for producing bacterial variation in the intestinal canal and self-purification of water when the latter is contaminated.

LABORATORY DIAGNOSIS

This consists in the isolation of E. coli from the urine in cases of genito-urinary tract infection, the blood in cases of septicæmia and purulent discharges from suppurative lesions.

Urine:—*Collection of the material:*—The sample of urine must be collected aseptically in a sterile vessel and carefully covered. In the case of female patients, it is essential to collect the sample of urine through a sterile catheter passed into the female bladder, after carefully cleaning the urethral orifice and the vestibule. In the case of males, *if a catheter cannot be passed*, the glans penis and the urethral meatus should be thoroughly disinfected and the patient asked to micturate. The first portion of the urine may be discarded or collected in a clean unsterilised pot for other examinations while the second portion collected in a sterile vessel for the requisite bacteriological diagnosis.

Examination:—The sample of urine should always be examined as to its physical, chemical and microscopic characters (*vide supra*). After these details are noted, a portion of the urine from the sterile vessel is centrifugalised and a MacConkey's bile-salt-lactose-neutral-red agar plate is inoculated with a loopful of the sediment. It is always good, in all cases, to inoculate a couple of nutrient agar tubes at the same time, as this will expedite the preparation of an autogenous vaccine, if the growth is pure and also to detect the presence of cocci, if present. After incubation for 24 hours, the typical rose-pink colonies will develop. Further studies may then be continued and the diagnosis definitely settled.

E. coli isolated from urine in cases of urinary tract infection should always be tested for its hæmolytic properties on blood agar or in a suspension of 5% red blood cells in peptone normal saline. This hæmolytic group of organisms forms a definite class by itself serologically and in other respects,

Purulent material:—In the case of a purulent material from other sources, a Gram stain should always be done and the bacterial flora examined. Then suitable culture media should be inoculated and the growth studied as above. In such cases, if associated pyogenic cocci are suspected, a plate of brom-cresol purple and ordinary nutrient agar plates should be inoculated.

Blood culture:—A word of caution may be said in connection with the isolation of E. coli from the blood in a suspected case of septicæmia or bacteriæmia. In cystitis, in pyelonephritis and in general blood infection, when a blood culture is desired by the physician, a special instruction should be given to the pathologist *to take the blood during the time of rigor.* At other times, the results are often unsuccessful. Another important point is the quantity of blood to be taken and it should never be less than 10 c.c.

Bacteriological examination of water for E. coli:—In examining samples of water bacteriologically for the presence of E. coli, it is very important to decide whether the strains of E. coli which are isolated are of fæcal origin or merely saprophytic organisms having nothing to do with fæcal pollution. To determine this important point, the Voges-Proskauer reaction, methyl red test, Koser's test and uric acid test (vide p. 89) should be carried out. The difference between the two is shown in the table below:—

	Voges-Proskauer reaction.	Methyl red test.	Koser's test.	Uric acid test.
Escherichia coli of fæcal origin	o	+	o	o
Escherichia coli of saprophytic origin ...	+	o	+	+

+ = Positive. o = Negative.

OTHER IMPORTANT MEMBERS OF THE COLON GROUP

These are quite a large number, existing both as parasites and also as saprophytes. They all produce luxuriant growths in ordinary nutrient agar with certain minor differential characters. They are differentiated mainly by their fermentative actions on sugars and by certain biological properties (*vide* p.p. 468-469).

Escherichia communior *(B. coli communior):*—It resembles Escherichia coli in all respects except that it *ferments saccharose* with the production of acid and gas. It occurs naturally in fæces and in materials polluted by fæcal excretions.

Escherichia acidi lactici *(B. acidi lactici):*—This organism is commonly present in milk, water and intestinal ca[illegible] man and animals, as a *non-pathogenic saprophyte*. It is a *non-mot[illegible]lus*, of the same size as the Escherichia coli and is Gram-negative. [illegible]ows in the usual laboratory media without any characteristic differe[illegible]n E. coli. It ferments the common sugars including lactose but has *no action on dulcite* and *saccharose* and in this respect, it differs from A. ærogenes (B. lactis ærogenes). It forms indol and coagulates milk with the production of acid.

Aerobacter ærogenes *(B. lactis ærogenes)*:—This is sometimes found in small numbers in the fæces but is mostly present as a *non-pathogenic saprophyte* in soil, sewage, decomposing vegetable matter, fruits and grains. It is often found in milk in association with a few others and is chiefly responsible for the ordinary souring of milk. It is a plump, non-motile bacillus of average length, possessing a faint capsule. It is Gram-negative. It grows vigorously in the ordinary media producing plenty of gas. The *colonies* on agar are big, convex and moist, with an opaque white colour and possessing a viscid consistency. It ferments all sugars, including lactose and *saccharose*, with the production of acid and gas but has *no action on dulcite*. It does not liquefy *gelatin*. The organism gives a *positive Voges-Proskauer reaction*, but *a negative methyl-red* test. It grows in Koser's medium.

Aerobacter cloacæ *(B. cloacæ)*:—This organism is widely distributed in nature, in the fæces of man and animals, as well as in sewage matter and other articles contaminated with fæces. It is a sluggishly motile bacillus of average size. It does not form any spore and is not capsulated. It does not retain the colour when stained by Gram's method (Gram-negative). The growth in ordinary media is luxuriant but without any great difference from Escherichia coli. The organism ferments all the ordinary sugars, including *lactose and saccharose*, but has *no action on dulcite*. It gives a *positive Voges-Proskauer reaction* but *negative methyl-red test*. It coagulates milk with the production of acid.

Proteus vulgaris *(B. proteus)*

Occurrence:—It is extensively present in nature, being always found associated with decomposing animal and vegetable matter, as well as in the soil and in fæces of men and animals. It is also found as a secondary contaminating organism in septic wounds.

Morphology:—It is a bacillus of usual size, sometimes occurring in small chains or filaments. It possesses numerous peritrichate flagella and is very actively motile. It has no capsule and does not form any spore.

Staining reactions:—It stains with all ordinary laboratory stains and is Gram-negative.

Cultural characters:—In nutrient broth, there is a uniform turbidity. On nutrient agar, the growth is very characteristic. There is a central main mass of growth, consisting of a greyish white translucent colony around which thin layers of bacterial growth creep out, forming irregular films in the surrounding areas. If the water of condensation in a nutrient agar is inoculated, the growth spreads like a thin film and covers the entire surface of the slope. This process of extension of growth has been designated as

" swarming." In *blood agar,* copious growth with hæmolysis and digestion of blood takes place. On *egg or serum media,* there is luxuriant growth with digestion and some liquefaction of the media.

Biochemical reactions:—The organism never ferments lactose and mannite but readily acts on glucose and saccharose, with the production of acid and gas. It rapidly liquefies gelatin and protein with digestion. This can take place only in the presence of oxygen. The methyl-red test and Voges-Praskauer reaction are both negative.

Serological reactions:—These are somewhat complicated as the Proteus group is heterogeneous, so far as its antigenic properties and immunity reactions are considered. Recently a strain, Proteus X 19, has been found by Weil and Felix (1916) to be agglutinated by the serum of typhus fever cases, the reaction being known as *Weil-Felix reaction.* Though the organism has no etiological relationship with the disease, still the reaction is so specific that it is of distinct value in the diagnosis of typhus fever. Pursuing their observation further, Weil and Felix noted two distinct types of colonies of Proteus X 19 on agar—the one with a spreading film, the H (*Hauch*) form and the other forming isolated colonies, the O (*Ohne-Hauch*) form. The former when injected into animals gives rise to both H (non-specific) and O (specific) agglutinins, while the latter produces only the specific small flaking O agglutinin. Moreover, the serum of patients suffering from typhus fever contains the O agglutinins which react specifically with O type of colony.

Toxin production:—No extracellular toxin is known, the poisonous substance being present inside the bacterial body.

Pathogenicity:—The organism or its endotoxin is very toxic to laboratory animals, subcutaneous or intraperitoneal inoculation causing death in a day or so. In man, Proteus vulgaris is mainly found as a secondary contaminating organism in various kinds of inflammatory lesions with ulceration. It is often found in cases of cystitis where the urine may contain even a pure culture of the organism. Sometimes, the ingestion of a large dose of bacilli, either with milk or with putrid meat, may cause symptoms of acute diarrhœa, specially in children.

Alcaligenes fæcalis (*B. fæcalis alcaligenes*). This organism is often encountered in fæces of normal persons. In certain pathological conditions of the large bowel, such as in late stages of bacillary dysentery, they are found in large numbers almost replacing the specific organism. It is an actively motile bacterium, possessing multiple peritrichate flagella. It is Gram-negative. The organism grows on ordinary culture media and is a *non-lactose fermenter.* Thus, in its morphological and cultural characters, it closely resembles typhoid bacilli. It differs from the latter in that it is *devoid of any fermentative properties in any of the sugar media* and produces a strong alkaline reaction in litmus milk. It does not form any indol, nor liquefies gelatin. The organism, as a rule, has no pathogenic property, but sometimes it has been isolated from the blood in cases of febrile illness, such as typhoid fever.

Table showing the cultural reactions of the colon group of bacilli.

Organisms.	Lactose.	Glucose.	Mannite.	Saccharose.	Dulcite.	Litmus milk.	Voges-Proskauer reaction.	Methyl red test.	Gelatin liquefaction.	Indol production.	Motility.
Escherichia coli (B. coli communis)	AG	AG	AG	o	AG	AC	o	+	o	+	+
Escherichia communior (B. coli communior) ...	AG	AG	AG	AG	AG	AC	o	+	o	+	+
Escherichia acidi lactici (B. acidi lactici) ...	AG	AG	AG	o	o	AC	o	+	o	+	o
Aerobacter ærogenes (B. lactis ærogenes) ...	AG	AG	AG	AG	o	AC	+	o	o	o	o
Aerobacter cloacæ (B. cloacæ) ...	AG	AG	AG	AG	o	AC	+	o	+	±	+
Proteus vulgaris (B. proteus) ...	o	AG	o	AG	o	AC	o	o	+	±	+

AG = Acid and gas; AC = Acid and clotting; + = Positive; o = Negative; ± = Variable.

Scheme for the identification of the Gram-negative intestinal group of bacilli.

Faeces

inoculated into

MacConkey's plate
(Bile salt-lactose-neutral red medium)

Colourless colonies
(Non-lactose fermenters)
Eberthella.
Salmonella.
Alcaligenes fæcalis.

Glucose

Acid but no gas
Eberthella.

Alcaligenes fæcalis.

Acid and gas
Salmonella.

Motility examined

The individual members of the group can only be differentiated by cross-agglutination and absorption tests.

Motile
E. typhi.

Non-motile
E. dysenteriæ.

Mannite

Acid and no gas
E. paradysenteriæ.
E. dysenteriæ, Sonne.
(Late lactose fermenter)

No change
E. dysenteriæ, Shiga.
E. dysenteriæ, Schmitz.

Formation of indol

Positive
E. dysenteriæ, Schmitz.

Negative
E. dysenteriæ, Shiga.

Pink colonies
(Lactose fermenters)
Escherichia, except Proteus vulgaris.

Dulcite.

Acid and gas
E. coli.
E. communior.

No change
E. acidi lactici.
A. ærogenes.

Saccharose

Acid and gas
E. communior.

No change
E. coli.

Saccharose

Acid and gas
A ærogenes.

No change
E. acidi lactici.

Note :—A. cloacæ (lactose fermenter) and Proteus vulgaris (non-lactose fermenter) are the only members producing liquefaction of gelatin.

CHAPTER XXVIII

LACTOBACILLUS

Acidophilic group or Lactic acid bacilli

(Gram-positive intestinal bacilli)

Under this group is included a number of bacteria which can withstand a degree of acidity fatal to most other bacteria and on this account have been termed "acidophilic." Though these bacilli are acid-resistant, they flourish better in a medium of average acidity, hence "aciduric" will be a better term than "acidophilic." These organisms are found in large numbers in the alimentary tract of various mammalian animals and are particularly prevalent during the stage of suckling. They constitute one of the most predominant intestinal flora of breastfed infants and of these, the two most important types are—(i) *Lactobacillus acidophilus* and (ii) *Bacillus bifidus.*

Lactobacillus acidophilus *(B. acidophilus):*—This is the most important member of the group. It occurs in large numbers in the fæces of breast-fed infants.

Morphology and staining—The organism is markedly pleomorphic, varying in length, form and arrangement. It may appear as a long stout bacillus (4 to 5 μ in length by 1 μ in breadth) and also as short coccal forms. Slender rods may be noted. It often occurs in long chains. It is non-motile and does not form spores. It is Gram-positive.

Culture—Cultivation may be done on agar plates containing whey and incubating aerobically at 37°C. The medium is prepared by adding a sufficient amount of 10 per cent. hydrochloric acid to skimmed milk heated to 80° to 90°C. and precipitating the casein. It is then filtered through cotton wool and the reaction is adjusted to pH 6·8 to 7·0. Peptone, 0·5 per cent. and agar, 1·5 per cent., are then added and the whole sterilised by autoclaving.

Colonies show in 48 hours and are very minute, thin and transparent and for their identification, they require to be magnified under the low power of the microscope. Two types of colonies are recognised, (i) one feathery in appearance and (ii) the other spindle-shaped with finger-like projections crab-colony). A convenient method of isolation of the organism from fæces is to inoculate the material into broth to which 1 per cent. acetic acid is added and incubated aerobically at 37°C. After this, subcultures can be made on whey agar plates.

Biochemical reaction—It ferments without gas production the following sugars, lactose, glucose, maltose and saccharose. In milk it produces acid and clotting.

Importance:—It has no pathogenic importance but considerable medical interest is attached to it because of (a) its rôle in souring of milk and (b) as a remedy (sour milk therapy) for certain chronic intestinal ailments.

ALLIED ORGANISMS

Lactobacillus bulgaricus (B. bulgaricus):—It is closely related to L. acidophilus, having the same morphological and cultural resemblance. It does not ferment maltose. The bacillus is chiefly found in sour milk in Bulgaria and Turkey. It is not normally found in the intestines of man. Regarding its value in sour milk therapy, it is less successful, as it is very difficult to implant the organism in the intestinal canal of man where it fails to grow.

Lactobacillus boas-oppleri (Boas-Opplei bacillus):—This organism is found in the stomach in conditions where hydrochloric acid is absent or deficient, as in gastric cancer, and specially with pyloric obstruction. Its characteristics are the same as those of L. acidophilus with the following differences:—

1. It does not ferment maltose and its acid production is less.
2. It grows best under micro-aerophilic condition.

Lactobacillus odontolyticus (B. acidophilus odontolyticus):—This organism was isolated from carious teeth and has been described by McIntosh and his co-workers as the cause of dental caries. It is a strong acid producer and ferments glucose, maltose, lactose and salicin. The acid produced by this organism is an important factor in the causation of the disease.

Döderlin's bacillus—This organism is an important member amongst the normal bacterial flora of the vagina. Being acidophilic, it helps to maintain the sterility of the vagina, specially of its upper two-third.

Bacillus bifidus *(Bacteroides bifidus):*—This is the predominant organism in the intestinal canal of breast-fed infants, particularly in the first few weeks of life. It is a Gram-positive, non-motile and non-sporing bacillus, of variable length. In anaerobic cultures, the organism shows bulbous or bifid ends from which it has derived it name. It grows poorly in the laboratory media and is an *obligatory anaerobe*. It ferments lactose, glucose, maltose and saccharose.

Sour milk and intestinal flora

Souring of milk is due to lactic acid fermentation which precipitates casein from the milk. Lactic acid is produced by the action of bacteria, belonging chiefly to the members of the Lactobacillus group, on the lactose of milk. Sour milk is a normal form of decomposition, resulting from breaking down of carbohydrates and is not harmful. Milk sometimes putrifies, due to the breaking down of proteins [illegible] spore-bearing bacilli. *Putrid milk* is alkaline and bitter, owing to the [illegible] of peptones; sometimes it contains toxic substances. Owing to [illegible] factors, the putrid milk is harmful.

In early infancy, the stool is acid in reaction and diet is rich in carbohydrates in the form of lactose; the chief bacterial flora being *lactic acid bacilli*. As age advances, the diet changes, the stool becomes alkaline in reaction and the bacterial flora also change. The place of lactic acid bacilli being taken up by *Escherichia coli* and other allied organisms which constitute about 60 per cent. of the fæcal flora.

The rationale of the sour milk therapy advocated by *Metchnikoff* is based on the fact that sour milk contains myriads of lactic acid bacilli which, when implanted in the large intestines, produce a high degree of acidity, a condition unfavourable and detrimental to the growth of other bacteria which are responsible for putrefactive processes. In this way, by inhibiting the putrefaction, it guards against "*auto-intoxication*" and has been used as a remedy for certain chronic intestinal ailments.

In this connection, it should be noted that *L. bulgaricus* is not a normal inhabitant of the human alimentary canal, particularly of children and some adults. Hence *L. acidophilus* is much more efficient for therapeutic purposes than the *L. bulgaricus*, as the former can readily be implanted in the intestinal canal.

Of recent years, it has been found that it is difficult to implant or to increase the numbers of these lactic acid bacilli by ingesting them by the mouth. Intestinal flora are controlled by diet rather than by bacteria ingested. It has been further discovered that the secret of sour milk therapy depends not only on the administration of the microbe but also on the modification of diet and ingestion of large quantities of lactose at the same time. It is by this means that the lactic acid bacilli can be definitely implanted in the intestine and become the predominant organisms.

KLEBSIELLA

Klebsiella pneumoniæ

Synonyms—*B. pneumoniæ*, *Pneumobacillus*, *Friedländer's bacillus*, *B. mucosus capsulatus*.

Friedländer, in 1883, isolated the organisms from the lungs of patients dying of pneumonia.

Habitat—A parasitic organism of the respiratory tracts of man.

Morphology—It is a short, thick, rod-shaped bacillus and usually arranged in pairs (diplobacillus). The organism is non-motile, non-flagellated and non-sporing. When seen in pathological exudates, it is found to possess a well marked *capsule*.

Staining reactions—It stains readily with ordinary aniline dyes and is Gram-negative.

Cultivation—The organism grows aerobically in all the ordinary laboratory media. In *agar*, it forms fairly large, raised, shiny, white colonies with viscid mucoid consistency (*cf.* A. ærogenes). In ***gelatin stab*** **culture,** it produces a characteristic nail-shaped appearance. The growth occurs as a white line along the stab and is heaped up at the surface to form the head of the nail. On *blood agar,* the organism grows with a wide zone of hæmolysis around its colony. In *broth,* there develops turbidity with a pellicle formation.

Biochemical reactions:—This organism is closely related to Escherichia coli. It ferments a number of *carbohydrates* with the formation of acid and gas. *Gelatin* is not liquefied and *indol* is only occasionally produced. *Voges-Proskauer reaction* is usually negative and *methyl-red test* is positive. Some of the strains grow on *Koser's medium.*

Serological reactions:—Like Diplococcus pneumoniæ, the organism can be classified into three main types (A, B and C) and a fourth heterogeneous group. The type-specificity depends, like the D. pneumoniæ, on the capsular substance.

Animal inoculation—When injected into mice and guinea-pigs either subcutaneously or intraperitoneally, it causes septicæmia and the capsulated organisms can be recovered from heart's blood.

Pathogenicity—Usually it does not play any pathogenic rôle. It has, however, been responsible for about 5 per cent. of the cases of lobar pneumonia and such cases are invariably fatal. The organism has been encountered in infection of the nasal sinuses, and has been isolated from empyema, appendicitis and suppurative processes of various kinds throughout the body.

ALLIED ORGANISMS

1. *Klebsiella rhinoscleromatis* (B. rhinoscleromatis)—This organism produces a slowly growing productive [illegible] of granulomatous inflammation (Fig. 243) on the mucous membrane of the nose, particularly at the external

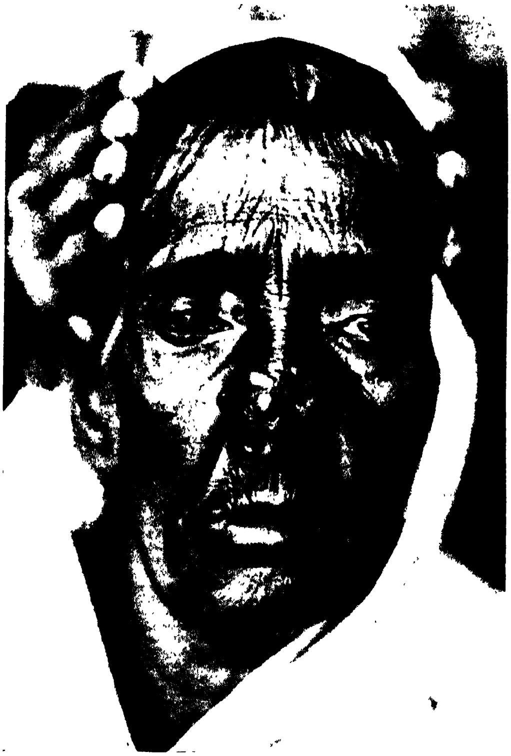


Fig. 243.
A case of rhinoscleroma.

This gives rise to mechanical obstruction of the air passages. On histological examination, the organisms are found in considerable numbers inside the large swollen cells which are known as the *cells of Mikulicz* (Fig. 244).

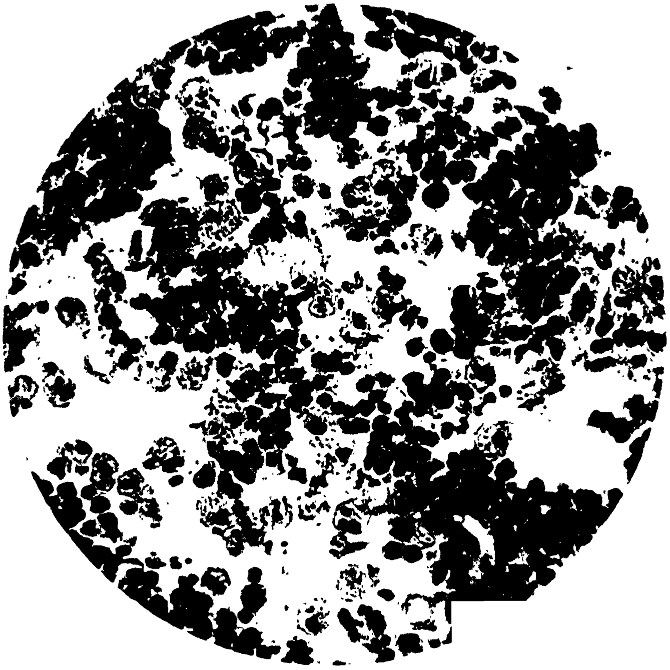


Fig. 244.
Photomicrograph of a section from rhinoscleroma. Note the large, clear cells with excentric and pyknotic nuclei. These are called Micalicz's cells. Besides these, there are masses of lymphoid cells.

2. *Klebsiella ozœnæ* (B. ozœnæ)—This is supposed to be the cause of fœtid nasal catarrh. By cultural methods and morphological examination, it is not possible to distinguish the organism from the other members of the group.

3. *Klebsiella granulomatis*—The organism appears to be related to granuloma inguinale.

CHROMOGENIC BACTERIA

The characteristics of these bacteria depend on their property of pigment production and they include the following:—

(1) Pseudomonas æruginosa (B. pyocyaneus)—Blue or green pigment.

(2) Serratia marcescens (B. prodigiosus)—Red pigment.

(3) Chromobacterium violaceum (B. violaceus)—Violet pigment.

Pseudomonas æruginosa (*B. pyocyaneus*)

Gessard, 1882.

Habitat:—This organism is frequently found as a harmless parasite upon the skin, in the upper respiratory tracts or in the gastro-intestinal tracts of man. It is frequently termed the bacillus of green or blue pus.

Morphology:—It measures about 1·5 μ to 3 μ in length. The bacillus is very actively motile, possessing one to three terminal flagella. It has no capsule and does not form spores.

Staining:—The organism stains readily with the usual aniline dyes and is Gram-negative.

Cultivation:—Ps. æruginosa is aerobic and facultatively anaerobic. It grows readily on the usual laboratory media and the optimum temperature for growth is 37°C. The growth is recognised by the characteristic greenish blue colour which diffuses through the whole substance of the medium. This is due to the production of a pigment, *pyocyanin*, by the organism and for which a free access of oxygen is essential. This pigment (pyocyanin) which is insoluble in water may be extracted from the culture medium with chloroform and crystallised out of such a solution. Besides this, another pigment, *fluorescin*, is produced by the organism, which is insoluble in chloroform but soluble in water.

On *agar*, the growth is confluent and appears on the surface as a moist, greyish, glistening layer. Very soon, i.e., after about 18 hours, the pigment diffuses into the medium, giving the characteristic colouration. In *broth*, the organism grows with the formation of a pellicle on the surface and a floccular deposit at the bottom. The whole broth also becomes turbid and slightly coloured, specially at the top. *Gelatin* is liquefied and *litmus milk* becomes clotted with the characteristic colouration.

Toxin:—The organism produces both endo- and exo-toxins. During its growth, the organism produces a ferment, *pyocynase*, which is exceedingly thermostable and has the power to dissolve certain bacteria, such as B. anthracis and C. diphtheriæ. A concentrated solution of this ferment (a broth culture filtrate which is evaporated *in vacuo* to one-tenth of its volume) is used for the purpose of protecting animals against anthrax infection and also used locally for removing diphtheria bacilli from the throat of convalescent cases.

Pathogenicity:—The organism is pathogenic to laboratory animals, specially guinea-pigs. The subcutaneous or intraperitoneal injection of 0·5 to 1 c.c. of a broth culture kills the animal in a day or two from toxæmia. There is also a severe local inflammation with necrosis and œdema produced locally at the area of the inoculation.

In human beings, its pathogenic power is comparatively slight. The organisms have been found as secondary invaders in certain suppurative conditions of the body, being responsible for the cause of "blue or green pus." The organism may, however, be the primary cause of infection, specially in ill-nourished children, causing diarrhœa and general infection, and in adults whose general health is poor. It is responsible for chronic otitis media and of some cases of gastro-enteritis in children. The organism has also been encountered in cases of empyema, broncho-pneumonia, ophthalmia, pericarditis, in blood in cases of septicaemia and also in genito-urinary infections.

Serratia marcescens *(B. prodigiosus)*—This is a minute Gram-negative bacillus which is found as a harmless saprophyte in water, soil and also in contaminated foodstuffs. It can be grown in ordinary culture media and the colonies are recognised by their characteristic red colour. It is devoid of any pathogenic property. The importance of this organism lies in the following:—

(i) It is used as a test organism to decide the size of the pores of a Berkefeld filter.

(ii) It is sometimes employed in anaerobic cultivation to absorb oxygen and create a condition of anaerobiosis (vide p. 382).

(iii) The organisms are sometimes used in the treatment of inoperable sarcomata and other malignant growths. For such a purpose, *Coley* used a vaccine of S. marcescens and virulent Streptococci, in doses of 0·05 c.c. Such a vaccine is often referred to as *Coley's fluid*.

BRUCELLA

Brucella melitensis

The causative organism of undulant fever.

Synonyms—*Alcaligenes melitensis; Bacterium melitense; Bacillus melitensis; Micrococcus melitensis.*

Bruce, in 1887, isolated the organism from the spleen of a patient who died of undulant fever and was able to reproduce the disease in monkeys. That the infection is transmitted by goat's milk was proved 18 years later (1905) by *Zammit* who was a member of the Mediterranean fever commission.

Habitat:—It is a strictly parasitic organism.

Morphology:—This is a minute organism and occurs singly, in pairs or even in short chains. Although definite bacillary forms are encountered (as in culture), it is more often cocco-bacillary in nature. It is a non-sporing and non-motile organism.

Staining:—It is Gram-negative.

Cultural characters:—It is an aerobe and can easily be cultivated in ordinary media but the growth is somewhat slow. It grows best at a temperature of 37°C. and preferably in an alkaline medium.

Agar—It grows slowly and takes about 2 to 4 days' time, sometimes even longer. Colonies are minute and transparent, like drops of dew. In old cultures, a brownish colour of the medium is noticeable and often crystals may appear.

Broth—Slight turbidity after 24 hours.

MacConkey's medium—No growth.

Litmus milk—Alkaline without coagulation.

Sugar media—No fermentative properties.

H_2S production—Nil.

Resistance:—The organism can withstand cold and drying for a long period without losing its vitality. Acid, produced by the souring of milk, has no effect on the organism. It is readily destroyed by heat and chemical antiseptics.

Toxin production—Whether any toxic product is formed by the organism has not been satisfactorily demonstrated.

Serological reaction:—Agglutinins are developed in the serum of patients suffering from undulant fever, as well as in the serum of infected animals (goats). This is utilised in the diagnosis of the disease during life (*vide infra*).

Animal inoculation:—The common laboratory animals, such as guinea-pigs, rabbits and mice, can hardly be infected except by intracerebral inoculation. For experimental work, monkeys are being extensively used, as in them the disease, exactly comparable to that in man, may be produced, either by feeding or by scarification of the skin.

Pathogenicity:—It is primarily a disease of goats in which the organism causes a septicæmic infection. Sometimes, a localised disease in the udder is also produced. On certain occasions, it may produce abortion in pregnant animals. The incidence of this caprine (goat) infection is very high (50 per cent.) in Malta. How the disease is transmitted from goat to goat is not yet definitely established.

In human beings, the organism produces the disease known as undulant fever, so called on account of its successive waves of pyrexia lasting for 2 to 3 weeks and alternating with periods of remission; this may extend over several months (3 to 4 months). The disease is also associated with enlargement of the spleen, secondary anæmia, transient painful swelling of the joints, orchitis and neuralgia. It is also called Malta fever or Mediterranean fever because of its common incidence in the shores of the Mediterranean and in its islands. The infection is conveyed by drinking goat's milk in which the organisms are excreted in large numbers. Such infected milk, however, does not show any gross physical or chemical alterations. As the organisms are not destroyed by the souring of the milk, infection may also occur from eating milk-preparations, such as butter and cheese. The infection has also been reported to occur through skin wounds and abrasions and cases are on record where laboratory workers were infected from handling cultures of Br. melitensis. The infection has also been known to occur in persons who handle infected milk. The disease has a low mortality, varying from 2 to 9 per cent. The organism gains entrance into the blood either through the digestive tract or through skin abrasions, producing bacteriæmia without any local lesions. *Post mortem,* no characteristic morbid changes could be detected, excepting an enlarged, soft and diffluent spleen. Brucella melitensis can be isolated in pure culture from heart's blood and splenic material.

Therapeutic considerations

Vaccine—The Mediterranean Fever Commission appointed in 1904 found that vaccination with dead cultures of Br. melitensis conferred a certain amount of protection to those who were exposed to infection. As a rule, an initial dose of 200 millions of organisms, followed later by a second dose of 400 millions, will suffice for the purpose.

Serum—Injection of an antiserum, obtained from convalescents from undulant fever, is found to have some specific effect in the course of the disease.

Prophylaxis:—This consists in destroying all the infected goats and as a personal protection to drink boiled milk in the endemic areas. As said above, prophylactic vaccination may be of some value.

Laboratory diagnosis

This consists of the following:—

(i) Isolation of the organism from blood, spleen puncture and less commonly from urine (10 per cent. of the cases). It has also occasionally been obtained from fæces. Blood culture is usually positive in the early stages of the disease, the spleen culture is positive at all stages and urine culture after the first fortnight of the disease.

(a) Film prepared from spleen puncture material, when stained by Gram's method, will reveal the characteristic Gram-negative cocco-bacillus.

(b) For a successful culture, at least 10 c.c. of blood should be taken and, during primary cultivation, a long incubation period, such as about a week, is necessary, before the growth can be detected.

(c) In the identification of the organism, agglutination reaction with stock sera should be resorted to.

(ii) Serum diagnosis—Agglutinins begin to appear in the blood serum after the fifth day of the disease and are of great diagnostic value after the first fortnight of the fever. Macroscopic agglutination is carried out in a series of ascending dilutions (1:25 to 1:1,000 dilution) against an emulsion of dead organisms and a titre of 1 in 100 may be taken as almost diagnostic. It should be noted that normal serum may agglutinate the bacilli in 1 in 100 dilution but this effect can be overcome by heating the serum to 55°C., which does not affect the specific agglutinins. Moreover, in patients suffering from undulant fever, the titre limit, like enteric fever, gradually rises and this will also confirm the diagnosis.

(iii) Burnet's intradermal test—This consists in the injection of 0·1 to 0·5 c.c. of the filtrate of a killed broth culture (*melitine*). A positive reaction is characterised by the appearance of a localised redness and œdema 6 hours after, at the site of inoculation.

(iv) Other accessory methods, such as a high mononuclear increase in the blood, may be of value.

Brucella paramelitensis (*Bacillus paramelitensis*)—A strain serologically different from Br. melitensis has been isolated from some cases of undulant fever. It has been suggested by some that it represents the rough variant of Br. melitensis.

Brucella abortus (*Bacillus abortus, Alcaligenes abortus*):—Discovered by Bang in 1897 in Copenhagen. The organism causes contagious abortion of cattle and swine by infecting the chorionic cells. In man it produces a disease indistinguishable from undulant fever (abortus type of undulant fever). The infection is obtained by drinking infected cow's milk or by handling infected animals. Br. abortus and Br. melitensis resemble each other so closely that it is difficult to differentiate one from the other except by absorption of agglutinin. There are two types—*porcine* (pig) and *bovine* (cattle)—of which the former is more related to Br. melitensis and is of greater virulence to human beings. The bovine type, however, cannot be grown under aerobic conditions and for its primary cultivation, it requires a micro-ærophilic condition, a high CO_2 content (10 per cent.) of the atomsphere being preferable. The presence of certain dyes inhibits the growth of Br. abortus but not of Br. melitensis. Thus, basic fuchsin (1 : 25,000) inhibits the growth of porcine type and thionin (1 : 30,000) that of bovine type. Production of H_2S by Br. abortus is another property which differentiates it from Br. melitensis. Another important point to be noted is that the inoculation of Br. abortus into a guinea-pig causes tubercle-like lesions in the spleen.

A strain, serologically different from Br. abortus, has been described and is called **Br. para-abortus.** This is supposed to be the rough variant of the former.

Brucella bronchisepticus (*Bacillus bronchisepticus*)—It is related biologically to the above two organisms and has been found as a secondary invader in canine distemper, a disease caus[illegible] filter-passing virus, and in which it is often responsible for the p[illegible]ary complication (broncho-pneumonia). It also causes broncho-pneu[illegible]n rodents. The organism is, however, motile and possesses 4 to 6 p[illegible]ate flagella.

PFEIFFERELLA

Pfeifferella mallei (*B. mallei* or *Glanders bacillus*)

The causative organism of glanders.

The organism was discovered by Loeffler and Schütz in 1882 from a horse dying of acute glanders.

Habitat:—It is strictly parasitic, being found chiefly in equines and man.

Morphology:—The organism is generally a straight or slightly curved rod, measuring about 2 to 5 μ in length and occurring singly. It does not form spores, has no capsule and is non-motile. Variation in size and form is often encountered. In cultures, long filamentous forms with lateral branching may occur; they are occasionally segmented and may have club-shaped extremities.

Staining reactions:—Pf. mallei stains readily with the usual aniline dyes and is Gram-negative. In tissues and in pus, the organism shows a granular or beaded appearance which, like C. diphtheriæ, is due to an inherent irregularity in the normal protoplasmic composition. It is not acid-fast.

Cultural characters:—Pf. mallei is aerobic but facultatively anaerobic and grows best at 37°C. It thrives on ordinary media but the addition of glycerin (5 per cent.) or small quantities of glucose into the media assists growth. The colonies are somewhat slimy and tenacious and have a brownish tint.

On potato slope—The growth is most characteristic and has certain features which are diagnostically important. No growth is usually visible within 3 days. After this period, there appear raised, translucent amber-yellow colonies, like drops of honey. With further incubation, the colonies coalesce, become opaque and reddish-brown and in eight days' time they turn chocolate brown.

In broth, it produces uniform turbidity and after a week's cultivation, a white scum begins to appear on the surface.

Biochemical reactions:—Glucose is the only sugar which is fermented with the formation of acid only but no gas. The reaction is very slow and may take as long as 2 to 3 weeks. In *litmus milk*, it produces slight acidification with late curdling.

Resistance:—Pf. mallei is not very resistant. Complete desiccation is often fatal in 24 to 48 hours. When exposed to strong sunlight, it is killed in 24 hours. Heating to 60°C. kills it in less than 10 minutes.

Action of germicides:—

1 per cent. carbolic acid kills it in 30 minutes.

3 per cent. carbolic acid, 1 in 5,000 bichloride of mercury and 1 per cent. solution of potassium permanganate kill it in 2 to 5 minutes.

It may survive for one or two months in water and this is an important practical point in connection with the spread of the disease through public drinking troughs.

Toxins:—The organism does not produce any exotoxin but an endotoxin can be easily obtained by extraction of the dead bacilli. This is known as *mallein*. One of its chief characteristics is its resistance, it being capable of withstanding temperatures of 120°C. and prolonged storage with but little loss of its potency.

Preparation of mallein:—Virulent organisms are grown in a 5 per cent. glycerin broth for 3 to 4 weeks. The culture is then sterilised either by steaming at 100°C. for 30 minutes or in the autoclave and then filtered. This filtrate is concentrated to one-tenth of its original volume by evaporating in a water bath.

Use:—Mallein is used in veterinary work in 1 c.c. doses to test the sensitivity of an animal to glanders infection. It has no effect when injected into a healthy animal. It should be remembered that mallein test, like the tuberculin reaction, is an example of allergic phenomenon. It is, however, not used in human beings for diagnostic purposes. Mallein may also be considered to be similar to "tuberculin" for the following reasons:—

(i) It is an endotoxin like tuberculin.

(ii) Its preparation is analogous to that of tuberculin.

(iii) Its diagnostic use is the same, i.e., to test the sensitivity to infection. The only difference is that mallein is exclusively used in veterinary work.

Serological reactions:—Infection with Pf. mallei results in the production of specific agglutinins in the blood of the infected animal and the titre may reach as high as 1 in 1,000. This is of considerable importance in the diagnosis of glanders infection.

Complement fixing bodies are also present in the immune sera.

Animal inoculation:—Of the laboratory animals, field-mice and guinea-pigs are remarkably susceptible and after subcutaneous inoculation, they die in a week or two, showing the following changes:—

(1) Caseation followed by ulceration at the site of inoculation.

(2) Enlargement of the proximal lymph glands.

(3) A considerable involvement of the internal organs, viz., liver, spleen and lungs which are studded with small yellowish nodules, resembling miliary tuberculosis.

Besides the above, a very characteristic lesion is developed when a male guinea-pig is inoculated intraperitoneally. The organism rapidly invades the tunica vaginalis where it seems to flourish best and within 3 to 5 days causes an acute suppurative inflammation (purulent orchitis) which is manifested externally by a swelling of the testicles. Post mortem, the two layers of the tunica vaginalis are glued together by a fibrino-purulent exudate. This reaction constitutes what is known as "Straus's reaction" and is used for the diagnosis of glanders infection, particularly in man. Death of the animal occurs in 2 to 5 weeks.

House-mice and white mice enjoy an almost complete immunity. Rabbits are less susceptible than guinea-pigs.

Pathogenicity

Glanders is an infectious disease pre-eminently affecting the horse, mule and the ass. Cattle are practically immune. The disease is also transmitted occasionally to other domestic animals and to man.

ANIMALS:—In horses, two forms of the disease are recognised, depending on the virulence of the infecting material and the susceptibility of the animal.

(1) Acute form (known as glanders)—This is limited to the nasal mucosa (septum nasi) and the upper respiratory tract, later involving the lungs and internal organs.

(2) Chronic form (known as farcy)—This involves the lymphatic vessels and glands forming multiple swellings.

Mode of infection:—This is not definitely known. It can certainly take place through superficial abrasions, either in the mucous membrane of the mouth and nose or in the skin. It is also believed to be the result of swallowing infective material through food and drink. Street water troughs and common mangers used by an infected animal serve to transmit the disease to healthy horses.

Pathology of glanders:—The organisms when ingested appear to pass through the intestines into the mesenteric lymph nodes and then to the lungs where they give rise to yellowish nodular growths. In a very heavy infection, however, such nodules may also develop in the internal organs, such as the lymph nodes, liver and spleen. In chronic cases, the lesions may remain localised in the lungs for a long time without any external manifestations of the disease. In acute cases, wider areas of the lungs may be involved, producing dark red, swollen, pneumonic consolidations. In advanced cases, the disease affects the mucous membrane of the upper respiratory tracts, viz., the larynx, trachea and the nasal septum and turbinate bones. The discharge from these lesions contains abundant bacilli and is highly infective.

In the skin, small nodules develop at the site of infection. These gradually get bigger and then soften and break down, leaving ragged indolent ulcers. These discharge a slightly blood-stained sero-purulent matter, containing a large number of bacilli. The regional lymphatic glands are enlarged, and undergo suppuration. Later on, multiple areas of suppuration may develop in the skin (farcy buds).

MAN:—Infection in human beings is rare and the disease is mainly occupational. It is usually contracted by those who are in habitual contact with horses and also with asses and mules. Thus, nearly all cases are seen amongst ostlers, grooms and coachmen.

The usual mode of infection is the contamination of a cutaneous wound or abrasion with the infected discharge from an animal. The disease is also known to have been contracted in the laboratory.

In man, it also occurs in acute and chronic forms. The lesions closely resemble those found in the horse but the disease usually runs a more acute course, producing signs and symptoms of pyæmia. Under such circumstances, abscesses are formed in the skin, muscles, joint cavities and the internal organs, such as the liver, spleen and lungs.

Therapeutic considerations:—Attempts to immunise animals by injections of vaccines prepared from dead or living organisms or of products of bacterial growth have not been met with success. Nor any effective anti-serum has so far been made to protect infected animals and man against the disease.

Laboratory diagnosis of glanders

This consists of the following:—

1. Microscopical examination.
2. Cultural examination.
3. Animal inoculation test (Straus's reaction).
4. Serological reaction.
5. Mallein test.

Microscopical:—Films prepared from pus or any discharge are stained with methylene blue and by Gram's method. The finding of somewhat beaded or granular, Gram-negative bacilli is suggestive. It should be remembered that in old lesions it is very difficult to find the bacilli.

Cultural:—The best medium for the purpose is a potato slope. The growth is recognised as amber-yellow colonies after 3 days' incubation, becoming chocolate-brown by the eighth day. Such a positive result is obtained when the culture is made direct from an unbroken nodule. With a mixed infection, however, cultures often yield a negative result.

In such cases, isolation of the organism may be possible by animal inoculation, since the organism can be obtained in pure culture from the tunica vaginalis testis of the inoculated guinea-pig.

Animal inoculation:—This is rarely required for the diagnosis of the equine glanders but is usually necessary for suspected glanders in man. A fully developed male guinea-pig is selected and an injection of 0·5 to 1 c.c. of the emulsion of the material is injected intraperitoneally. In 3 to 5 days, the Straus's reaction (swelling of the testicles) is observed.

Serological reaction:—Agglutination test has been used for the diagnosis of glanders in horses. The technique is the same as that for the Widal's reaction in typhoid fever. It is preferable to use an emulsion of dead culture for personal safety. Serum dilutions are made from 1 in 100 to 1 in 4,000 and the tubes incubated for 24 hours at 37°C. Agglutination reaction occurring in high titre, such as 1 in 1,000 or more, should only be accepted as significant of infection.

Complement fixation test has also been used for diagnostic purposes and it has been regarded as more reliable than the agglutination reaction.

Mallein test

It is used to diagnose glanders infection in an animal. Routes of inoculation are subcutaneous, ophthalmic and intradermopalpebral. A *subcutaneous injection* of 1 c.c. of mallein in the skin of the neck produces in the infected animal the following changes:—

(1) Local reaction—Formation of a large and painful inflammatory swelling, reaching a diameter of 5 inches in 24 hours.

(2) General reaction—Within 6 to 8 hours, there is a sharp rise of temperature—1·5 to 3°C. above normal.

(3) Focal reaction—The affected lymphatic vessels or farcy buds become swollen.

The subcutaneous mallein test has ceased to be used as a practical method for the field diagnosis of glanders, having been supplanted by the following two tests.

The ophthalmic test:—A conjunctival reaction may also be elicited which appears to be more convenient and reliable. About 3 drops of concentrated mallein are dropped into one of the eyes of the animal. A positive reaction is manifested by swelling of the eyelids and a purulent secretion from the tested eye. The reaction usually commenees within 5 or 6 hours and lasts from 24 to 36 hours.

The intradermo-palpebral test:—About 0·1 c.c. of 1 in 4 concentrated mallein is injected into the dermis of the lower eyelid. A typical reaction appears after 10 to 12 hours and is characterised by an extensive œdema involving both the upper and lower eyelids so that the eye is almost closed. There is also an acute conjunctivitis.

Pfeifferella whitmori (B. whitmori)

A rare tropical disease known as melioidosis. resembling glanders and affecting man, has been observed in Burma and the Malay States. It is principally an infection of rats and rodents amongst which it occurs as an epizootic. The disease is communicated to man from these animals and is characterised by a long continued fever of a septic type, broncho-pneumonia, abscess formation in the internal organs and a fatal termination. The causative organism (Pf. whitmori) was first isolated by Whitmore from a glanders-like disease (melioidosis) in human beings in Rangoon in 1913.

In 1921 Stanton and Fletcher gave the name melioidosis to this disease and B. (Pf.) whitmori to the causative organism.

Pf. whitmori resembles Pf. mallei in certain respects but differs from it in many others.

Morphologically—These two organisms are fairly similar with the exception that Pf. whitmori is actively motile and frequently shows bipolar staining.

Culturally—Pf. whitmori grows more rapidly and luxuriantly than Pf. mallei. On MacConkey's agar, Pf. whitmori grows freely, forming red colonies (being lactose fermenter) while Pf. mallei entirely fails to grow.

Biochemically—Pf. whitmori on first isolation is an active fermenter of sugars, producing acid but no gas in glucose, lactose, maltose, saccharose, dulcitol and mannite.

Serologically—The two organisms are almost identical.

Pathogenically—Both the organisms are able to reproduce typical Straus's testicular reaction on intraperitoneal injection into a male guinea-pig.

CHAPTER XXIX

HÆMOPHILUS

The generic name, "Hæmophilus," was given by the society of American Bacteriologists, because of the fact that the presence of hæmoglobin in the medium is an essential requirement for the artificial cultivation of these bacteria. This group includes the following:—

A. Those affecting the respiratory tracts:—
   1. *Hæmophilus influenzæ* (B. influenzæ or Pfeiffer's bacillus).
   2. *Hæmophilus pertussis* (B. pertussis; or Bordet-Gengou bacillus of whooping cough).

B. Those affecting the conjunctiva:—
   3. *Hæmophilus conjunctivitidis* (Koch-Weeks bacillus of acute infectious conjunctivitis)—This is now regarded as identical with H. influenzæ.
   4. *Hæmophilus lacunatus* (Morax-Axenfeld bacillus of subacute infectious conjunctivitis or angular conjunctivitis).

C. Those affecting the genital tracts:—
   5. *Hæmophilus ducreyii* (Ducrey's bacillus of soft chancre).

HÆMOPHILUS INFLUENZÆ

(B. influenzæ or *Pfeiffer's bacillus of influenza.)*

Historical:*—Pfeiffer,* in 1892, observed these organisms in the sputum of patients suffering from influenza and was successful in obtaining them in pure culture by cultivating in a medium containing hæmoglobin. He regarded the organism as the etiological agent of influenza.

Morphology:—The influenza bacillus of Pfeiffer is an extremely minute organism, measuring about 0·5 μ by 0·2 μ. The organisms are found either singly or in pairs, being arranged end-to-end, or most commonly in clusters, but never in chains. They do not form spores, have no capsules and are non-motile. In artificial cultures, there is marked pleomorphism and the organisms may show various shapes and sizes.

Staining reactions:—The organism, unlike other bacteria, is difficult to stain with the usual aniline dyes. The most successful stains are dilute carbol fuchsin (1 in 10 dilution of Ziehl-Neelsen's stain) for 5 to 10 minutes or Loeffler's methylene blue for 5 minutes. The best results are obtained if the smear is first treated with dilute acetic acid and then the stain is applied. The bacilli may show bipolar staining, the ends taking the stain more deeply than the centre which takes the stain less intensely (*cf.* Pasteurella pestis). It is Gram-negative.

Cultural Characters:—The organism is an aerobe and grows best at or near the body temperature (37°·5C). It cannot be cultivated in ordinary nutrient agar and the usual blood agar medium is also not very satisfactory. The addition of altered blood (boiled or digested), however, greatly promotes the growth of the organisms. The following media (p. 70) are very suitable for this purpose:—

(1) Boiled blood agar (cooked blood agar or chocolate agar).

(2) Agar containing peptic digest of blood (*Fildes* medium).

X and V factors:—Unlike all other bacterial species, the influenza group of bacilli requires for their permanent cultivation, the presence of certain accessory growth factors in artificial media. Of these, two have been recognised and are named as X and V factors. These have no nutritional effects but are accessory food factors comparable to vitamins in mammalian physiology.

The *X factor* is associated with methæmoglobin or hæmatin and may be obtained from the hæmoglobin of any species of animal, although it is also associated with the iron-containing substances present in fruits, potatoes and other vegetable materials. This factor is thermo-stable, remaining unaltered even after autoclaving at 120°C., and a very minute amount is sufficient for the purpose. Its action is of the nature of a catalytic agent, accelerating the transfer of atmospheric oxygen to the organisms which under normal circumstances are unable to utilise such oxygen directly. For this purpose they employ only the intermediate peroxides with the help of a catalyst (X-factor).

The *V factor* is present in fresh animal and vegetable tissues including blood, fresh potato, yeast, etc. It is thermo-labile, being destroyed at a temperature of 100°C. and a much larger quantity of the substance is required for growth. The mode of action of the V factor is not definitely known.

Character of the colonies:—Within 24 to 48 hours, colonies are just visible to the naked eye and they appear as very minute transparent circular dots—like "drops of dew."

Sometimes the organism can be cultivated on ordinary blood agar medium, when grown in symbiosis with pyogenic cocci. If a blood agar plate is inoculated with influenza bacilli and staphylococci, many fairly large colonies of the former are found in the neighbourhood of the colonies of the latter. Such a *'satellite' growth* of H. influenzæ in symbiosis with pyogenic cocci is very characteristic.

Biochemical reactions:—It has no proteolytic action, neither does it liquefy serum or gelatin. Indol formation is variable, as some strains produce it while others do not.

H. influenzæ does not produce any characteristic fermentative reaction in sugar media.

All influenza bacilli have the power to reduce nitrates to nitrites.

Hæmolytic activities of H. influenzæ are variable. There are some strains of this group of organisms, which produce marked hæmolysis of red blood cells, while there are others which do not cause any such change.

Resistance:—Influenza bacillus is an extremely delicate organism. It is very sensitive to heat, desiccation and weak antiseptics; thus, outside the body it cannot live for any length of time. Direct sunlight kills it in 3 to 4 hours and in dried sputum it dies within one to two hours. A temperature of 60°C. acting for 5 minutes will kill the bacilli. Weaker antiseptics, such as phenol 2 per cent., produce instantaneous death.

In cultures, the vitality does not persist for more than a few days; the organism dies quickly, unless it is frequently subcultured, say every 4 to 5 days.

Toxin production:—The nature of the toxin is not definitely known. An emulsion of the organisms, either dead or alive, when injected into the peritoneal cavity of a rabbit or guinea-pig, causes death of the animal with petechial hæmorrhages on the peritoneal and pleural surfaces.

Serological reactions:—Up till now, no definite serological tests are known which can be utilised as diagnostic methods for the disease. The blood serum of patients and immunised animals may agglutinate the bacillus but, owing to the presence of several strains, the phenomenon is inconstant and variable. Complement fixation tests have been described but they do not, in any way, materially help the serological analysis of the influenza bacilli.

The blood serum of convalescent patients and immunised animals also contains certain antibodies, such as bacteriotropins and bacteriolysins.

Animal inoculation:—The common laboratory animals cannot be infected experimentally with the organisms.

Pathogenicity:—No animal except man acquires influenza under 'natural' conditions. Since the time of Pfeiffer (1892), this organism has been found to be intimately associated with the disease known as 'clinical influenza.' It has been suggested that infection usually occurs in the upper respiratory tracts where the bacilli may produce an acute catarrhal inflammation. Subsequently, the infection may extend into the lower respiratory tracts, involving the lungs and producing bronchitis and bronchopneumonia. In very severe cases, the organisms may gain entrance into the general blood stream. The bacilli may be found in large numbers in the nasopharynx, as well as in the sputum.

Rarely, the organisms may be isolated from the blood in cases of septicæmia and from the cerebrospinal fluid in cases of meningitis.

In the upper respiratory tracts, the H. influenzæ may cause a more or less chronic inflammation which may spread into the neighbouring structures, producing sinusitis, otitis media, conjunctivitis and other ocular complications. A bacteriological examination of the discharge from these areas often reveals a pure culture of the organism.

Although H. influenzæ has been constantly found in cases of '*clinical influenza,*' the true etiological relationship of the organism with the disease has not yet been firmly established. Whether the organism is the primary infecting agent or it is only a secondary invader, being superimposed on an already inflamed respiratory mucosa brought about by an as yet undetermined factor, is still an open question. In these cases of 'clinical influenza,' other secondary micro-organisms, such as D. pneumoniæ, Str. hæmolyticus and N. meningitidis, are also found along with H. influenzæ and it is the infection by the secondary organisms, which determines the severity of a case of influenza.

Note:—In 1921, *Olitsky* and *Gates* cultivated an organism, *Dialister* (Bacterium) *pneumosintes*, anaerobically in Smith-Noguchi's medium from filtered naso-pharyngeal washings of cases of influenza during the earliest stages of the illness.

Therapeutic considerations:—Owing to the absence of exact knowledge about the etiological agent of influenza, specific vaccines do not offer any material help. The vaccine which is used for prophylaxis is a mixed one, consisting of H. influenzæ (100 millions), D. pneumoniæ (100 millions), Streptococci (40 millions) and N. catarrhalis (100 millions). Two inoculations are given at an interval of ten days, the second dose is twice that of the first. The idea of giving such a vaccine is to raise the resistance against the secondary infections which are so harmful as compared with the "primary invader," and when regarded in this light, it appears rational.

Considering that the fatality of a case of influenza is due to streptococcal septicæmia, early administration of anti-streptococcal serum in very bad cases is worth trial.

Diagnosis:—The diagnosis of the disease mostly rests with the clinicians and finding of large numbers of H. influenzæ by microscopical and cultural examinations in a case of the respiratory type, is a very valuable aid. Leucopenia which is a feature of the disease may be of some value in the diagnosis.

HÆMOPHILUS PERTUSSIS *(Bacillus pertussis)*

Bordet-Gengou bacillus of whooping cough.

In 1906, *Bordet* and *Gengou* cultivated the organism from the sputum of whooping cough patients.

Morphology:—Hæmophilus pertussis bears a considerable morphological resemblance to H. influenzæ with which it is frequently associated. It is a small ovoid bacillus without any capsule. It does not possess any flagella, is non-motile and does not form any spore.

Staining reactions:—It is Gram-negative. Bordet and Gengou recommended the phenol-toluidine blue staining solution with which the bacilli are best stained. When stained with this stain, the bacilli show polar staining.

Preparation of the stain:—

Toluidin blue	5 gms.
Alcohol	100 c.c.
Water	500 c.c.

Dissolve and add 500 c.c. of a 5 per cent. solution of carbolic acid in water. Let this stand for one or two days and then filter it.

Cultivation:—It is aerobic and grows well at a temperature of 37°·5C. Accessory growth factors (X and V) are not required by the H. Pertussis which can grow without them.

The best medium for the primary cultivation of this organism is blood agar but growth may be obtained in ordinary laboratory media after repeated subcultures. *Special medium* (p. 71) used for the isolation of the organism is Bordet-Gengou medium (Glycerine-potato-blood-agar).

Character of the colonies:—The colonies become visible to the naked eye after 48 hours' incubation at 37°C., when they appear as moist, raised, opaque areas of pearly-white lustre, surrounded by a zone of hæmolysis.

Biochemical reactions:—It does not ferment any carbohydrate, has no liquefying action on proteins, does not form indol and has no reducing action on nitrates.

Toxins:—Endotoxins are only produced.

Serological reactions:—Injection of the organisms into an animal is followed by the formation of agglutinins and complement-fixing bodies in the blood.

Pathogenicity

ANIMALS:—Whooping cough has never been produced experimentally in animals. Intravenous or intraperitoneal injection of autolysed cultures will usually kill a rabbit within 24 to 48 hours by toxic effects.

MAN:—In human beings, it produces the disease whooping cough, characterised by catarrh of the respiratory tracts (the catarrhal stage), followed later by periodically recurring laryngeal spasms (paroxysmal stage). Whooping cough is essentially a disease of childhood, having a special tendency to attack young infants. The disease is transmitted from person to person by *"droplet infection."* The droplets of sputum, disseminated by a coughing patient, contain large numbers of bacilli which gain entrance into the respiratory tracts. The disease is most infective during the catarrhal stage before the whoop develops.

Lesions:—The organism does not produce any gross lesions. The principal feature of the disease is some laryngitis and bronchial catarrh, characterising the initial catarrhal stage of the disease which lasts about a week or thereabout. Later the characteristic whoop develops and the patient enters into the second or paroxysmal stage which lasts for about 5 to 6 weeks. The organisms which are deposited on the throat and air tubes rapidly multiply and destroy the cilia or mechanically interfere with their functions. As a result, the secretions accumulate in the air passages, leading to irritation which excites the spasmodic cough.

Certain lung complications may develop in the course of whooping cough. One of the common complications is broncho-pneumonia which is usually caused by secondary organisms present in the throat; the usual invading agent being Streptococcus hæmolyticus.

Another very important complication which may occur during the convalescent period is the flaring up of a latent tuberculous focus (acute miliary tuberculosis) in whooping cough patients (children) unless they are properly looked after.

Owing to the strain of coughing, certain complications arise, such as subconjunctival and other hæmorrhages, appearances of herniæ, prolapse of rectum and also myocardial degeneration.

Therapeutic considerations:—Debré (1926) is of opinion that convalescent **serum,** obtained from patients in the fourth week, when used in small amounts, such as 3 c.c., can protect contacts.

The majority of workers believe that the *prophylactic* use of a pertussis **vaccine** confers a certain amount of immunity against an attack and even those who may contract the disease get it in a milder form. *Madsen* recommends three subcutaneous injections of a vaccine, containing 10,000 millions of organisms per c.c., at intervals of 4 days. The doses are 0·5, 0·7 and 1·0 c.c. respectively. *Bloom,* on the other hand, recommends the use of a mixed vaccine, containing H. pertussis, 5,000 millions and H. influenzæ, 3,500 millions per c.c. The vaccine employed should be a fresh one and not more than 10 days old. Three subcutaneous injections of 1 c.c. of the vaccine are given on alternate days and the immunity established is maintained by injecting 1 c.c. of the vaccine every second year.

A pertussis vaccine, when used for *curative purposes,* no doubt lessens the severity of the paroxysmal stage and shortens its duration. In order to get the maximum benefit, it is advisable to use the vaccine therapy as early as possible. Bloom (1925) recommends the use of a freshly prepared mixed vaccine, same as that used for prophylactic purposes. Doses of 1 c.c. are given on alternate days until the prominent symptoms disappear. Then the vaccine is given on the third or fourth day.

Prevention of whooping cough:—This consists of the following:—

(1) Use of a prophylactic vaccine before the attack. This has already been described.

(2) *Isolation of the sick:*—The period of quarantine depends on the probable duration of infectivity. Whooping cough is infectious during the catarrhal and early paroxysmal stages and the bacteriological investigation of whooping cough patients shows that the bacilli are found in considerable numbers during the first fortnight of the cough. The bacilli then disappear completely in most cases during the fifth an[illegible]th weeks. This can be observed from the following data given by [illegible] *Kristensen* (1927) about the frequency of the presence of H. pertuss[illegible]tients at different stages of the disease.

Stage of the disease.	*Per cent. positive.*
Catarrhal stage	75
Paroxysmal stage, 1st week	57
,, ,, 2nd week	61
,, ,, 3rd week	45
,, ,, 4th week	40.5
,, ,, 5th week and later	9.0

Thus, to control the spread of infection in a large number of cases, the ideal method will be to isolate the patient for a period of 5 weeks from the commencement of the whoop and to discharge him when the paroxysmal cough has ceased for a fortnight. Before releasing the patient from quarantine, it would be an additional safeguard to make use of "release cultures," i.e., if three successive cultures from the throat prove sterile, the patient can be set free.

Laboratory diagnosis:—The diagnosis of whooping cough in its catarrhal stage is a very difficult problem and it is practically impossible unless the characteristic whoop is heard. The one which is of assistance in the early diagnosis is the presence of lymphocytosis (15,000 to 50,000 per c.mm.) in the patient's blood. For the bacteriological diagnosis, however, the following measures are resorted to—microscopical, cultural and serological examinations.

1. **Microscopical**—In the early stages of the disease, the H. pertussis may be present in large numbers in the sputum but it is not possible to identify it with certainty from a simple microscopical examination of a stained film.

2. **Cultural**—Successful results are obtained by this method in the catarrhal stage and the first week of the "whoop."

The thick viscid pellets which are voided at the end of a paroxysm or the invisible droplets which come out during the act of coughing, are the best materials for cultural purposes as they come out from the depth of the respiratory tract and contain plenty of bacilli. Nasal and buccal secretions often yield a negative result. Two methods may be employed for obtaining samples for cultural examination.

First method—In this procedure, a fit of cough is induced by depressing the tongue of the patient with a spatula. The little pellet of expectoration which is voided at the end of the paroxysm is collected on a sterile gauze or cotton wool. This is then washed several times in sterile saline and inoculated on to the special medium. This method is a difficult one as it requires the help of an experienced person to obtain the samples.

Second method—This consists in holding a plate of special medium with lids removed, for a quarter of a minute, in front of the patient's mouth, during the paroxysm of cough. The plate is kept at a distance of 4 to 6 inches from the patient so that he does not actually spit but coughs on to the plate. By this procedure, the invisible droplets of sputum (containing numerous bacilli) come out of the depth of the respiratory tract and are caught on the plate.

After sampling, the plate is returned to the laboratory as early as possible and incubated at 37°C. for two days. The growth can not be seen before two days, after which the plate is examined for the characteristic pearly colonies of H. pertussis. A film is then prepared from the suspected colony, stained by Gram's method, using dilute carbol fuchsin as the counterstain, and examined for the characteristic organism.

3. **Serological**:—*Agglutination reaction* is less reliable for diagnostic purposes. The test may however be of some value when a complete and prompt reaction occurs with a dilution of 1 in 100 to 1 in 200 of the patients' serum.

Complement fixation test, as mentioned before, can not be utilised for diagnosis in the early stages. During the later stages of the disease and after the attack is over, confirmation of the diagnosis can be obtained by this

HÆMOPHILUS CONJUNCTIVITIDIS

(Bacillus of Koch-Weeks.)

This micro-organism which is the cause of acute contagious conjunctivitis was first described by *Koch* (1883), while he was in Egypt. Later, *Weeks* (1887), in New York, noted the same organism and was able to cultivate and reproduce the disease on human conjunctiva by inoculation.

This organism is identical with H. influenzæ which it resembles morphologically and culturally and in staining reactions. It is not possible to distinguish the two bacilli except perhaps by direct inoculation into the human eye. Koch-Weeks bacillus will produce an acute conjunctivitis while Pfeiffer's bacillus will fail to produce any.

Diagnosis of acute infectious conjunctivitis:—

1. *Microscopical:*—This consists in making a smear from the conjunctival secretion and staining it by Gram's method. The organisms are very fine, small and Gram-negative.

2. *Cultural:*—The bacilli cannot be grown in ordinary agar. Cultivation on blood agar is absolutely necessary. Colonies resemble those of H. influenzæ.

HÆMOPHILUS LACUNATUS

(Morax-Axenfeld bacillus.)

The organism was first described by *Morax* in 1896 and *Axenfeld* in 1897.

Morphology:—It is a short, thick bacillus, about 2 μ in length and occurs in pairs (diplo-bacillus), but also singly or in short chains. The organisms are usually found free in the secretions and only occasionally inside the leucocytes and epithelial cells. The organism is non-motile, nonsporting and non-capsulated.

Staining:—It is readily stained with ordinary aniline dyes and is Gram-negative.

Cultivation:—As the organism cannot be cultivated in ordinary media, such as agar, gelatin or broth, the addition of blood or serum or any other native animal protein is necessary for its cultivation. When cultivated on Loeffler's serum medium, the colonies appear as small indentations ("pits" or "lacunæ"), indicating liquefaction of the medium. The specific name, "*H. lacunatus,*" was given to the bacilli from this characteristic growth.

Animal inoculation:—The organism is non-pathogenic to animals, but in man a typical conjunctivitis may follow when inoculated into the conjunctiva.

Pathogenicity:—The organism produces a catarrhal inflammation of the conjunctiva, specially noticeable in the angles of the eyes and most severe at or about the caruncle *(angular conjunctivitis)*. It usually attacks both the eyes and runs a subacute or chronic course.

Diagnosis:—This is usually settled by demonstrating the characteristic organism in the conjunctival discharge by microscopical and cultural examinations.

OTHER CLOSELY ALLIED ORGANISMS

These are characterised by their ready growth on ordinary culture media and consist of the following:—

1. *Diplobacillus liquefaciens:*—The organism was met with in certain forms of conjunctivitis.
2. *Bacillus duplex non-liquefaciens:*—The organism was found in corneal ulcers and sometimes in the sputum of acute and chronic bronchitis.
3. *Zur Nedden's bacillus:*—This was isolated from severe corneal ulcers with hypopyon.

HÆMOPHILUS DUCREYII

(Ducrey's bacillus of soft chancre.)

Ducrey, in 1889, described the presence of this micro-organism in the discharges from soft chancre. He was also successful in transferring the disease by inoculation methods.

Morphology:—It is a small bacillus, appearing singly, in pairs, in small groups or rarely in short chains (streptobacillus). It is non-motile, does not possess flagella and does not form spores. Involution forms may occur.

Staining reactions:—It is stained with ordinary aniline dyes and frequently shows polar staining. It is Gram-negative.

Cultivation:—It cannot be grown in ordinary media and requires a medium containing blood. It is very difficult to obtain primary cultures from the lesion or from the secondary bubo. The best way to accomplish this is to inoculate the infective material into tubes containing clotted rabbit's blood which has been heated to 55°C. *(vide infra)*. Subcultures may then be made on blood agar plates and after 48 hours' incubation, discrete, small, transparent and grey colonies appear, surrounded by a slight zone of hæmolysis.

Pathogenicity:—It has not been transmitted to animals but can be experimentally inoculated into man.

The infection is conveyed from one individual to another by direct contact. The organism produces an acute inflammatory reaction, usually upon genitals or in the surrounding skin. After a short incubation period of 24 hours, the lesion appears as a small pustule which rapidly ruptures, leaving irregular ulcers with undermined edges and necrotic floor. These ulcers are usualy multiple. Sometimes, owing to secondary infection, the ulcer assumes a destructive, phagedenic and rapidly spreading character. Usually, the infection spreads to the neighbouring lymph nodes, giving rise to abscess formation, commonly spoken of as the "bubo." Thus, *soft chancre* or *chancroid* is a venereal affection.

Therapeutic considerations:—A specific **vaccine** has been prepared and its use is claimed to have a curative effect on the lesions.

An **antiserum** has also been obtained by inoculating rams intravenously with dead and living cultures of Ducrey's bacillus. Two or three intramuscular injections of 10 c.c. each, given every fourth day, produce a marked beneficial effect upon chancres and buboes, resulting in their rapid healing.

Laboratory diagnosis

1. **Microscopical**:—Examination of a stained smear may help to a great extent if the characteristic chains of small Gram-negative bacilli are found.

2. **Cultural**:—*Teague and Deibert* (1920) have elaborated a simple technique for direct diagnostic cultivation. The method is as follows: Into a series of small test tubes is distributed sterile rabbit's blood, in amounts of 1 to 2 c.c. The blood is allowed to clot at the room temperature and is then heated to 55°C. for 5 minutes to destroy the bactericidal substances. The material from the chancroidal sore or bubo is inoculated into the serum around the clot. After 24 hours' incubation, smears are made from the serum and stained by Gram's method. If the characteristic chains of small Gram-negative bacilli be found, it indicates a positive diagnosis for Ducrey's bacillus. It has been mentioned that 90 per cent. of true chancroids can be diagnosed by this culture method.

3. **Cuti reaction**:—A specific cutaneous reaction is obtained 48 hours after the injection of 1 c.c. of a bacillary emulsion (using a control at the same time) which may be regarded as diagnostic of the disease.

CHAPTER XXX

THE COMMON COLD

Definition:—The term, "common cold," although difficult to define accurately, usually comprises a group of transient symptoms with mild febrile reactions and is characterised by varying grades of catarrhal inflammation of the upper respiratory tracts. The main symptoms may be summarised as follows:—

There is usually a history of exposure to cold or to unhygienic, confined and over-crowded environments, such as opera and cinema houses. This is followed by headache, malaise and fever, varying from 99°F. to 102°F., accompanied by pains and aches in the body. Constipation, scanty high coloured urine, loss of appetite and insomnia are often very marked. The patient invariably suffers from troublesome sneezing, coughing, hawking and a feeling of choking sensation in the nose, with irritation and profuse watery discharge from it. There may be some redness of the eyes with probably mild conjunctivitis. Some degree of sore throat, with or without huskiness of voice, may be present and in children, there may be an acute earache.

In an uncomplicated case, the symptoms last for 2 to 3 days, after which the patient begins to feel better. The irritation of the nose with watery discharge is replaced by a profuse, thick, yellowish, muco-purulent material and there may be some cough with expectoration of similar matter from the throat.

Etiology:—Common cold is known to be due to a catarrhal inflammation of the mucous membrane of the nose and nasopharynx caused by bacterial infection. The micro-organisms which are usually associated with such a condition are *Diplococcus pneumoniæ*, *Streptococcus hæmolyticus*, *Streptococcus viridans*, *Hæmophilus influenzæ*, *Neisseria catarrhalis*, *Staphylococcus pyogenes aureus*, and rarely *Corynebacterium diphtheriæ* and *Neisseria meningitidis*.

It has also been thought to be due to a *filterable virus* which acts as the primary agent in starting the inflammation and the above micro-organisms are then superimposed only as secondary invaders.

Pathogenesis of common cold:—The mucous membrane of the upper air passages and particularly of the nose, possesses certain peculiarities of its own. It consists of a very highly vascular structure with a submucosa equally rich in vessels and lymphatics, the whole being under the control of a very sensitive nervous mechanism. Its vascularity is easily altered by reflex nervous stimuli derived from distant parts of the body and also from various external and physical influences. For instance, a variable temperature, high degree of humidity, wet cold ground, breeze saturated with cold rain and foggy weather, always lead to the alteration in the mucous membrane of this place, making it more prone to a catarrhal inflammation. It has been definitely found that when a person lives inside a badly ventilated room, containing warm and stagnant air, the mucous membrane of his nose gets swollen, hyperæmic and covered with a thick secretion. On the other hand, if the ventilation is good and the air is cool, the mucosa remains pale, contracted and dry.

An occupant, on passing out of an ill-ventilated room to the open-air, subjects his nasal mucous membrane to a good deal of sudden vascular alteration which acts as a great strain. This accentuates the liability to catarrh. Another important factor which often works in common cold is that in a congested, badly ventilated room, the head remains warm but the feet are cold, owing to the floor level being cooler than the head level. This also reflexly leads to much congestion and swelling of the nasal mucosa. This sensitiveness of the mucous membrane of the nose varies very widely in individual subjects, some persons being highly susceptible, due to a congenital condition. Heredity plays some part as this susceptibility is often found to run in families. Certain constitutional diseases may also predispose to this condition and this is specially the case with rickets.

It should always be remembered that common cold, whatever may be the predisposing factor, is due to an infection. The latter is usually obtained from another infected individual, either during coughing, sneezing and similar acts or through fomites. Crowded, ill-ventilated rooms, such as cinema and opera houses, theatres, meeting places, etc., are particularly dangerous, not only for inducing a pathological alteration of the nasal mucous membrane but also for their bacteria-laden atmosphere and the consequent risk of infection. The actual development of the catarrh depends on two factors, (i) the number and virulence of the micro-organisms which attack the mucous membrane and (ii) the local as well as the general resistance of the individual. A heavy dose of infection may be received either by a direct hit from a coughing bout or by staying too long in air heavily laden with the offending germs.

Many of the bacteria mentioned above may normally inhabit the mouth, nose and throat without showing any evidence of disease. This may be due either to a lowered virulence of the organisms or to sufficient local and general immunity of the host. But whenever this equilibrium is lost and there is a loss of resistance and lowering of vitality by exhaustion, overwork, exposure, intoxications, such as heavy drinking and smoking, convalescence from other illnesses, and gastro-intestinal derangements, the bacteria are able to penetrate the mucous membrane and produce their pathogenic effects. In a mild case, a temporary invasion of the mucous and submucous tissues occurs, with its accompanying reactions, gi[illegible]ise to the symptoms of "*Common cold,*" "*Coryza*" or "*Clinical inf*[illegible]

If, however, the infection is virulent and the resistance of the individual is insufficient, the invasion may not remain limited to the submucosa but may extend to the blood stream, producing a preliminary septicæmia with secondary localisation in the lungs and meninges, causing lobar pneumonia, broncho-pneumonia and meningitis respectively. In some cases, owing to an abnormally high grade of virulence of the invading organisms and very low vitality of the patient, the mucosal reactions may be slight and even negligible but the blood-infection and secondary complications become prominent from the very beginning. The authors have seen cases which complained of trifling malaise and slight fever, developing into fulminant meningococcal septicæmia and dying within 24 hours.

Relationship of common cold to the spread of epidemic diseases:—"Common cold" is a very important factor in the outbreak and spread of many epidemic diseases. The act of sneezing and coughing and the expectoration of individuals suffering from "cold," result in the promiscuous scattering of the infecting bacteria, as well as many other serious bacterial agents, such as *N. meningitidis, C. diphtheriæ, D. pneumoniæ,* etc., for which these patients may have been acting as carriers. Thus, they not only spread the virus that has given them the "colds" but also scatter the above-mentioned virulent micro-organisms responsible for serious epidemic diseases.

Some important places from which the infection may spread are badly ventilated schools, asylums, hospitals, military camps, ships and other vessels on the stream, factories, cinemas and theatres and crowded meetings. In fact, any place where large numbers of men are assembled in a small compass.

Some facts about treatment:—"*Prevention is better than cure*" should be the maxim. This can be achieved by the following :—

(1) Educating the public by popular lectures and demonstrations.

(2) Avoiding intimate contact, such as kissing, sleeping in the same bed, sneezing and coughing. Care should be taken of all fomites, including used handkerchiefs.

(3) Infected children should not attend school, nor should such teachers undertake their teaching work.

(4) Disinfection of the nose, throat and all fomites.

Everybody should remember the beneficial effects of open air, sunshine and a cool dry atmosphere. Warm feet and a cool head are always ideal. When taking a warm bath, one should always wash his head with cold water. Exposure to cold humid atmosphere, specially after a drinking bout, should be prohibited. Exercise in the open air always keeps the circulation active and the body fit. Schools and institutions should be built in such a way that a thorough ventilation may be assured. Segregation of the infected persons is very essential. To maintain the body resistance at its maximum level, rest in bed and freedom from worry should be insisted upon.

The question of *prophylactic inoculation* naturally presents itself. There is much authoritative opinion that a vaccine composed of the usual catarrh producing organisms does confer some protective power. In the case of a person who suffers from cold once or twice a year but remains quite well during the intervening period, four to six fairly large doses of a mixed catarrhal vaccine given before the expected time of the attack may prevent the onset of the "cold" in the next season. The vaccine should contain various strains of *D. pneumoniæ, Streptococcus, Staphylococcus, H. influenzæ, N. catarrhalis* and *Klebsiella pneumoniæ* (Friedländer's pneumo-bacillus). Usually, stock vaccines from the market are used and 1 c.c. should contain 1,000 millions of each organism. A dose of 0·2, 0·4, 0·8 and 1 c.c. may be injected at weekly intervals successively.

In the case of a person who suffers from repeated "colds" and is presumably a carrier, the "colds" being exacerbations of a latent infection, an autogenous vaccine is always preferable. The doses should be small and gradually increased. A high degree of temporary immunity may be obtained, although the infection may persist.

Although the prophylactic vaccination protects the individual for short periods, it certainly gives some relief. The attacks are lessened in intensity and the onset of the serious complications, such as suppuration in the sinuses, extension to the lungs and blood stream, is prevented.

CHAPTER XXXI

PASTEURELLA

Under this group are included all the closely allied bacilli causing "hæmorrhagic septicæmia" in various animals as well as in man. Though the individual bacilli differ in their cultural and other minor characters, they possess common species characteristics which bring them into one group. They are as follows:—

(i) *Short and thick bacilli.*

(ii) *Non-motile.*

(iii) *Polar staining.*

(iv) *Decolorisation by Gram's method of staining (Gram-negative).*

(v) *Inability to liquefy gelatin.*

(vi) *Marked pathogenicity for animals.*

Classification:—

A. *Pathogenic for man and animals.*

1. **Pasteurella pestis** (B. pestis)—Producing plague in man and septicæmia in animals, particularly in rodents.

2. **Pasteurella tularensis** (B. tularensis)—Producing plague-like disease in animals and typhoid-like condition in man.

B. *Pathogenic for animals only.*

3. **Pasteurella pseudotuberculosis** (B. pseudotuberculosis rodentium) —Formation of tubercle-like nodules in rodents.

4. **Pasteurella septica** (Trevisan)—This includes a variety of organisms producing hæmorrhagic septicæmia (Pasteurelloses) in various animals. A specific name has been given to each strain, corresponding to the animal from which it is derived.

From fowls—*Past. aviseptica* (Bacillus of chicken cholera).

From pigs—*Past. suiseptica* (Bacillus of swine plague).

From cattle—*Past. boviseptica* (Bacillus of hæmorrhagic septicæmia in cattle).

From rabbits—*Past. lepiseptica* (Bacillus of rabbit septicæmia).

From sheep—*Past. oviseptica* (Bacillus of hæmorrhagic septicæmia in sheep).

PASTEURELLA PESTIS

(Bacillus pestis)

The organism of Oriental plague.

HISTORICAL:—The plague bacillus was isolated by *Kitasato* and *Yersin* (1894) from human patients during an epidemic of plague at Hong-kong.

MORPHOLOGY:—

Size and shape:—The organism is a short, thick, oval bacillus with rounded ends (cocco-bacillary form), measuring about 1·5 μ by 0·7 μ.

Arrangement:—It usually occurs singly or sometimes in pairs; chain formation (strepto-bacillary form) is found in fluid (broth) culture only (Fig. 245).

Involution forms *(Pleomorphism)*:—This is the most noticeable feature of the organism and is particularly seen in old cultures and old lesions. The organisms become markedly swollen, stain faintly and assume globular, pear-shaped, elongated or irregular forms—more resembling a culture of yeast than of bacteria (Fig. 246). In young cultures, formation of involution forms can be hastened by growing the organism in "salt-agar" (nutrient agar to which 3 to 5 per cent. of sodium chloride has been added).

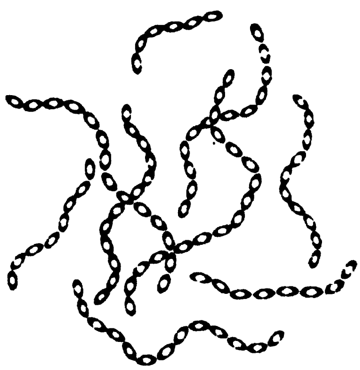


Fig. 245.

A smear from a broth culture of *Past. pestis*, showing the arrangement of organisms in chains.

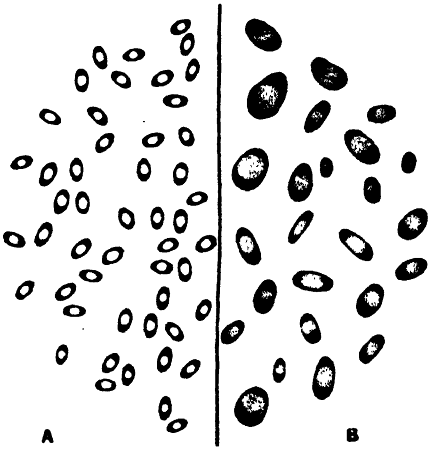


Fig. 246.

Pasteurella pestis.
A—Normal forms; B—Involution forms.

Capsules:—*Rowland* (1914) has confirmed that the plague bacillus possesses a well-defined capsule which contributes to the sliminess of its culture on solid media. The presence of a capsule, however, is very inconstant.

The organism is *non-motile* and *non-sporing*.

STAINING REACTIONS:—The organism is stained readily with the *ordinary aniline dyes*. Dilute carbol-fuchsin and methylene blue are most frequently employed. With these stains, the bacillus shows the characteristic polar staining, *i.e.*, central part of the bacillus is unstained or but faintly stained while the ends take the stain deeply. This is an important feature in its identification. When stained by *Gram's method*, it is Gram-negative. When stained with *Leishman's, Giemsa's or Unna pappenhiem's stain*, the bipolar staining is very nicely demonstrated.

CULTURAL CHARACTERS:—Although it is an aerobe, it grows best under anaerobic conditions or under reduced oxygen tension. Unlike other pathogenic micro-organisms, the optimum temperature for its cultivation is about 30°C. instead of 37°C.; but growth occurs quite satisfactorily at body temperature. The most favourable reaction of the culture media is neutrality or weak alkalinity, though slight acidity does not prevent growth. It grows readily in any of the ordinary media but the addition of 0·1 per cent. blood, 10 per cent. serum or 0·05 per cent. sodium sulphite to nutrient agar produces a luxuriant surface growth under ordinary aerobic conditions.

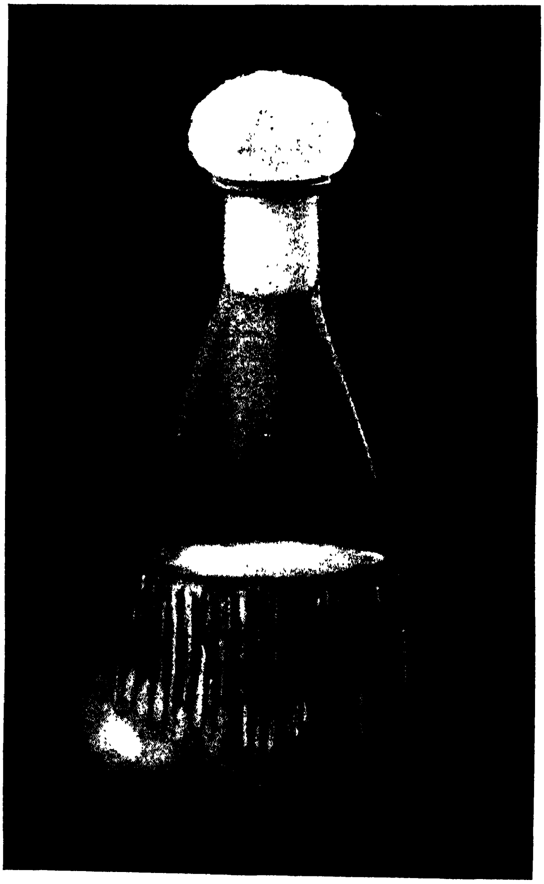


Fig. 247.
"Stalactite" growth of *Past. pestis* in ghee broth.

On agar, the colonies are small, semi-transparent and glistening; the growth is sticky and tenacious.

In broth, the organisms grow slowly. They sink to the bottom or adhere to the walls of the tube where they form floccular or granular deposits, leaving the upper part clear. If oil or melted butter is floated on the broth, the organisms grow downwards from the lower surface of the supernatant oily layer in the form of long delicate filaments composed of masses of bacilli (in about a week). This is what is known as the "*stalactite*" growth (Fig. 247) of *Hankine* and to secure this, care should be taken to incubate the flask in a place where shaking or vibration can be prevented, as the slightest movement is sufficient to detach the threads which are very fragile.

Note:—Past. pseudotuberculosis rodentium also gives such "stalactite" growths.

On MacConkey's medium:—The presence of bile salts does not inhibit the growth of Past. pestis.

BIOCHEMICAL REACTIONS:—

Fermentation reactions:—Glucose, lævulose, maltose and mannite are fermented with the production of acid without gas. Lactose, saccharose and dulcite are not fermented.

Litmus milk—No change.

Peptone water—No indol is produced.

Gelatin—No liquefaction.

Hæmolysis—Past. pestis is non-hæmolytic.

Methyl red test—Positive.

Autolysis:—This takes place particularly in fluid culture. It is not marked towards the beginning of a growth but occurs only in old cultures.

VIABILITY:—The organisms are not very hardy nor are they well adapted for a saprophytic existence in nature. They are readily destroyed by heat, desiccation and antiseptics. They withstand, however, a considerable degree of cold, surviving a temperature of 0°C. for 40 to 100 days. Direct sunlight destroys them in 4 to 5 hours. Drying by artificial means kills them rapidly. Heat, either dry or moist, is very injurious to them. A broth culture, when heated to 55°C. for 15 minutes, is completely sterilised.

The organisms have poor resistance to the action of antiseptics. For instance, a 5 per cent. phenol or 1 in 1,000 $HgCl_2$ kills them in 10 minutes.

Another very important point in this connection is that the growth of the organism in living tissues is rapidly inhibited by those of other pyogenic bacteria. *Post mortem*, the bacilli are completely overgrown by the usual putrefactive micro-organisms and killed within 3 to 4 days.

In *culture*, the bacilli can retain their viability for months, if kept in a cool dark place with sufficient moisture in the medium.

VIRULENCE:—Normally, plague bacilli are highly virulent to experimental animals. Their virulence may be intensified by passage through susceptible animals and attenuated by repeated subcultures in artificial media and by other artificial methods. In nature, the virulence of the organism diminishes with the decrease of an epidemic as shown by the fall in the case-mortality. In artificial culture, they usually retain their virulence for months and years, specially if some native protein is added to the medium and provided the latter contains plenty of moisture and is kept in a cool dark place.

TOXINS:—No true exotoxins but endotoxins only are present. These are liberated after disintegration of the bacilli. The toxins elaborated by Past. pestis damage the vascular endothelium, causing hæmorrhages in the skin, mucous and serous surfaces and various internal organs. It is also an intense tissue poison, producing cloudy swelling and fatty change in various organs.

SEROLOGICAL REACTIONS:—Agglutinins and other antibodies are developed in the serum of inoculated animals and infected persons. All the strains are agglutinated by the specific serum but such a reaction cannot be utilised for the identification of the organism because of its auto-agglutinating property. Immune sera have been prepared by injection into horses and this can be used for therapeutic purposes.

ANIMAL INOCULATION:—Laboratory animals, particularly **guinea-pigs** and **white rats,** are very susceptible. *Subcutaneous inoculation,* even of a small dose, results in the production of a local lesion, characterised by congestion, œdema and necrosis. Enlargement of the proximal lymph nodes and signs of a general septicæmia develop, producing intense congestion of all the internal organs. On *post mortem* examination, the spleen is found to be enlarged and often studded with greyish white nodules, resembling miliary tuberculosis. This gives a stippled appearance, as if "dusted over with pepper." Greyish white necrotic areas surrounded by a zone of intense congestion may also be seen in the liver and lungs (Fig. 248). Large numbers of bacilli are found in smears from all these lesions.

Other methods of inoculation:—

(i) Smearing the *conjunctival* or the *nasal mucous membrane* with a culture of the organisms results in infection. In this case, a cervical bubo results, followed by the usual picture of septicæmia and death.

(ii) Rubbing the freshly *shaved skin of the abdomen* with a culture—The ability of Past. pestis to pass through the intact shaven skin has been regarded as very characteristic. It appears that the organisms gain entrance into the body through minute abrasions which are produced in the process of shaving.

PATHOGENICITY

IN ANIMAL:—Many animals are liable to contract plague under natural conditions and the disease is characterised by the enlargement of the lymphatic glands and spleen and rapid death from septicæmia. The disease occurs in an epizootic form amongst rats and various rodents which play an important rôle in the spread of the disease. *Post mortem,* the characteristic changes are:—

(1) Dark red, subcutaneous injection of the abdominal wall.

(2) Swelling of the neck-glands with œdematous, hæmorrhagic infiltration of the surrounding tissues. (The flea prefers to inhabit the skin of the neck).

(3) Changes in the spleen, liver and lungs are exactly similar to those mentioned under experimental inoculation.

A chronic form of lesion is sometimes produced, particularly towards the end of an epidemic, in which many encapsulated thick-walled abscesses, containing the bacilli, are found in the areas corresponding to the sites of the primary buboes and also in the spleen and liver, without seriously affecting

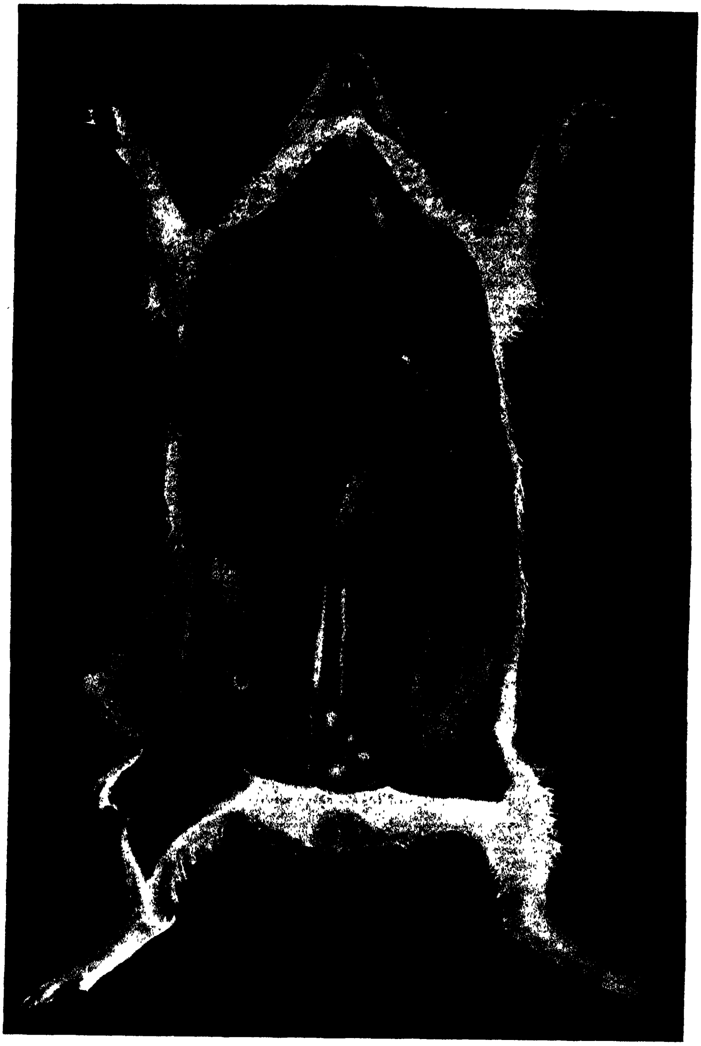


Fig. 248.

Post mortem appearances of a guinea-pig injected subcutaneously with *Past. pestis*. All the organs are intensely congested. The spleen is enlarged and studded with greyish white nodules. Liver and lungs show areas of necrosis surrounded by a wide zone of congestion.

the health of the animal. Although under such conditions, the disease is latent, the organisms are potentially virulent to other animals. It has been suggested by some authorities that this may be a factor in keeping up the disease, but there is no definite evidence in favour of such a hypothesis.

The infection in the rat takes place usually by the bite of an infected flea.

IN MAN:—The disease is endemic in parts of Asia; it periodically becomes epidemic and occasionally pandemic. Human plague may assume one of the three clinical types: (a) Bubonic, (b) Pneumonic and (c) Septicæmic.

Bubonic plague:—This forms by far the commonest type of cases in India. The organism enters the body through the skin by the bite of an infected flea, where it may form a localised lesion, producing a primary vesicle. The adjacent lymph nodes, depending on the seat of the primary infection, are enlarged and these are called "primary buboes." The commonest glands involved are the groin glands but axillary and cervical glands may also be the sites of primary buboes. Usually, one group of glands is affected first, constituting the primary bubo and later on, other groups become enlarged. Along with this glandular enlargement, there is marked pyrexia and great prostration. An intense septicæmia results before death.

Pathology:—The swollen *lymph nodes* are intensely inflamed and show widespread necrosis and hæmorrhages. The surrounding connective tissues are also involved and are œdematous and hæmorrhagic. The primary bubo is thus composed of a group of enlarged glands fused together and embedded in a mass of œdematous and hæmorrhagic connective tissue. In the secondary buboes, the periglandular inflammation is absent and the hæmorrhagic character is not so marked. In the early stages, the plague bacilli are found in large numbers in a film preparation made from the gland pulp. But with the degenerative changes in the glands, the number of the bacilli diminishes and involution forms appear.

The *spleen* is enlarged and shows many areas of hæmorrhagic necrosis. A smear from the splenic pulp will show a large number of bacilli.

Secondary lesions, in the form of hæmorrhages and necrosis, are found in the lungs, liver and other internal organs. Liver and kidneys show a marked cloudy swelling and fatty change. Heart muscle is severely affected, showing fatty degeneration.

Petechial hæmorrhages are found on all the serous surfaces, particularly the pericardium and pleura.

Pneumonic plague:—This is a very serious condition and is invariably fatal. There develops an acute broncho-pneumonia of a hæmorrhagic type. The expectoration consists of blood-tinged mucus which soon becomes bright red. It contains large numbers of bacilli. This type is highly infectious as the infecting organisms are conveyed from the diseased person to healthy contacts directly through droplets of sputum which the patient sprays out during cough.

Pathology:—After inhaling the infective particles, the bacilli are deposited on the mucous membrane o^f^ the upper respiratory tract and the bronchi. The organisms start an intense inflammation of the wall of the tubes through which they penetrate and affect the contiguous lung tissue. They also pass downwards to reach the capillary tubes where a similar type of inflammation is produced and from which the corresponding lung area is involved. Thus the earlier lesions are broncho-pneumonic (Fig. 249) but as the disease advances, several such areas may coalesce forming big areas of consolidation. The picture may then resemble the lesions of lobar pneumonia. As the toxins act severely on the capillary endothelium, there are extensive hæmorrhages with comparatively few leucocytes in the alveoli. Unlike lobar pneumonia, there is little or no fibrin but marked œdema. There may be much enlargement of the bronchial glands.

The organism multiplies in the lesions with enormous rapidity and the sputum resembles a pure culture of the bacilli. The latter rapidly pass into the general blood stream causing a septicæmia.

Fig. 249.

of plague broncho-

Septicæmic plague:—Primary septicæmic cases are very rare. Both the bubonic and the pneumonic forms rapidly become septicæmic. The organisms multiply in the blood stream with such an astounding rapidity that in many cases, particularly before death, they may be demonstrated in the peripheral blood while doing a differential count of leucocytes. According to the Plague Research Commission (1906 and 1908), 1,000,000 bacilli per c.c. were often found in a patient's blood 20 hours before death and 10,000 organisms per c.c. were not uncommon findings.

TRANSMISSION OF PLAGUE

Plague is primarily a disease of the rats and other rodents, such as ground squirrels in California and tarabagan, a species of ground moles in Manchuria, and man is only secondarily infected. An epizootic in those animals is followed by a plague epidemic in human beings. The infection, in *bubonic plague*, is spread by fleas, and it is through their agency that the disease is transmitted from rat to rat and from rat to man. In *pneumonic cases*, however, the transference is direct by droplets of sputum.

Relation of Plague to rats and fleas

Rats:—In India, the reservoir of the *Past. pestis* is the large grey sewer rat, the Rattus (Mus) norvegicus or decumanus (Fig. 250). An epizootic of plague first occurs in these rats from which the disease is transferred to the smaller black house rat, Rattus (Mus) rattus (Fig. 251). The parasitic insects, fleas, act as intermediaries in the transmission of the disease from one animal to another and also to man. After the death of the animal, when the body temperature falls, the fleas begin to desert it and are in search of a suitable victim, in the absence of which they bite men, usually in the leg or foot. Sweepers who are in the habit of picking up dead rats (which may remain still warm) are bitten in their hands and arms. Dead rats which are cold are less dangerous to handle as the fleas [illegible] already left them. It has been observed at the Haffkine Institute, B[illegible] that by recording and

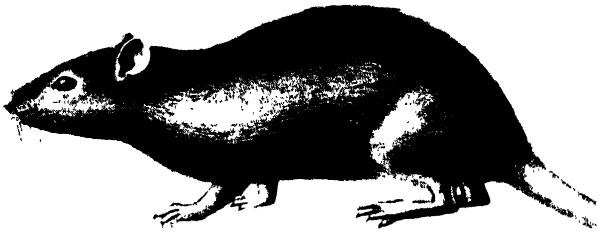


Fig. 250.

Rattus norvegicus.

This is the brown sewer rat. Note the small ears and stout tail which is never longer than the combined length of head and body.

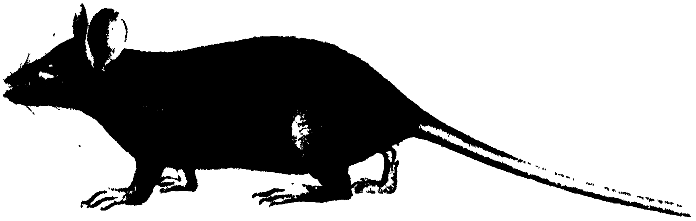


Fig. 251.

Rattus rattus.

This is the black house rat. It is small in size, weighing about half that of R. norvegicus. Note the large ears and thin tail which is longer than the combined length of head and body.

constructing graphs of the infection in rats, an extension to human beings can be foretold. Human epidemic usually follows 2 to 3 weeks after an epizootic in rats.

Fleas (Fig. 252):—These are laterally flattened wingless creatures, about 2 to 3 m.m. long. They are blood sucking insects and are numerous and active during winter months and this doubtless accounts for the seasonal prevalence of bubonic plague. Temperature favourable to the multiplication and activity of the flea ranges from 21° to 25°C. Higher temperatures and a dry atmosphere kill the flea vector and therefore reduce the incidence of the disease during the hot weather in India. Leaping ability of the adult flea is 3 to 5 inches, never exceeding 6 inches, and it can hop 30 yds. on legs. The fleas concerned in the transmission of plague are the following:—

1. *Xenopsylla cheopis*, the Indian rat flea. This has been found by the Plague Commission to be the principal vector of plague in India.
2. *Ceratophyllus fasciatus*, the common rat flea of Europe and North America.
3. *Pulex irritans* (human flea)—It may transmit Past. pestis under experimental conditions but plays little or no part in the natural spread of the disease.

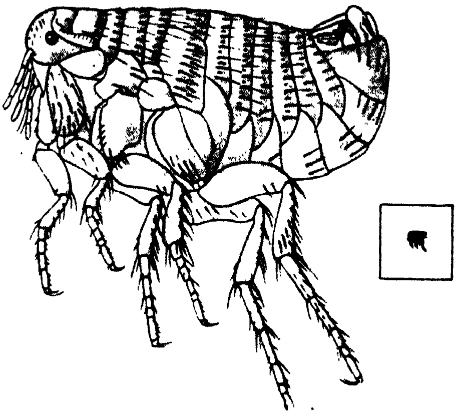


Fig. 252.
Xenopsylla cheopis.
The common Indian rat flea which is an important transmitting agent of Oriental plague. The natural size of the flea is shown in the inset.

Mode of infection (Fig. 253):—The fleas act as passive, intermediate carriers of the bacilli. They also serve as multipliers of the germs. When a flea bites an infected rat, it sucks some blood into its œsophagus and proventriculus. The bacilli remain alive and multiply to such an extent as to occlude the alimentary canal at the entrance to the stomach. Fleas in this condition are able to suck blood by the pumping action of the pharynx. When next it bites a human being, it regurgitates and cleans the proventriculus before it can obtain blood and in this way the bacilli are injected into the wound produced in the hosts by the bites of the flea. The bacilli are also present in the flea's excreta, and by scratching the site of the bite on which the insect has defæcated, the bacilli may be forced into the wound and enter the body. But this mode of infection is not the common one.

Bacilli may retain their virulence in the alimentary canal of a flea for 3 to 4 weeks and hence, the latter remains infective for a long period. It has been found by the Indian Plague Research Commission that the infectivity of a rat flea fed on a plague infected rat is maximum during the first 5 days after which it gradually diminishes but may last as long as two to three weeks.

Fleas do not play any part in the dissemination of Pneumonic plague. The infection seems to be acquired through the respiratory tracts from man to man.

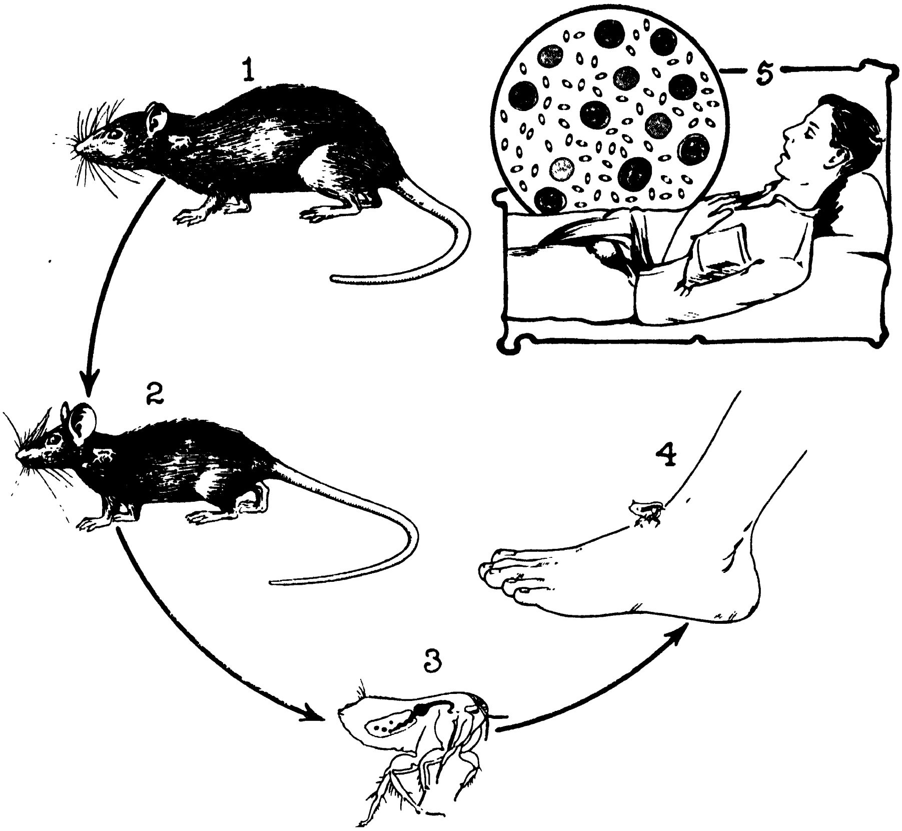


Fig. 253.

Mode of infection of Oriental plague.

1—Rattus norvegicus (large, brown, sewer rat), the first victim
2—Rattus rattus (small, black, domestic rat), the second victim
3—Xenopsylla cheopis, the intermediary agent in the transmission of rat to rat and rat to man.
4—The infected rat flea biting the leg of a human being.
5—A case of bubonic plague, showing inguinal bubo. In the m film preparation of the material from gland puncture, s staining bacilli, lymphocytes and a few red blood corpuscles.

THERAPEUTIC CONSIDERATIONS

Serum:—*Yersin and Lustig* have advocated that intravenous administration of anti-plague serum, in doses of 100 c.c. during the first two days, offers very promising results. Recent studies have shown that serum prepared from calves and sheep are much more potent than that obtained from horses.

Vaccines:—These are mostly used for prophylactic purposes and confer an immunity which lasts for 6 to 12 months or more. The vaccine is prepared by cultivating the organism by Haffkine's method, i.e., by favouring stalactite growth. This is shaken every few days and after this, the whole flask is incubated at 25°C. for 6 weeks. It is finally sterilised by heating to 65°C. for one hour and phenol 0·5 per cent. is added for preservation. The dose varies from 0·5 to 4 c.c.

Note:—It has been shown recently that Past. pestis contains two different antigens, one heat-stable somatic antigen and the other capsular antigen which is heat-labile at 100°C. and is well developed when the culture is made at 37°C. Immunising value of a vaccine is high when it contains this capsular antigen.

Prophylaxis:—This consists of the following:—

1. Compulsory notification.
2. Isolation of the sick.
3. Quarantine.
4. Preventive inoculation.
5. Destruction of fleas by the use of pulicides, such as kerosene oil, naphthalene, etc.
6. Rat elimination by building rat proof houses.
7. Rat destruction by fumigation with hydrocyanic acid gas, sulphur dioxide, etc.
8. Wearing face masks when attending cases of pneumonic plague and gloves for handling bubonic cases.
9. Evacuation of houses and infected villages during an epidemic.

LABORATORY DIAGNOSIS

The diagnosis of plague rests mainly on the demonstration of the causative organism.

Material collected for a ***bacteriological examination*** will vary according to the type of the disease. Thus in bubonic plague, the material is taken from gland puncture, in pneumonic plague from sputum and in septicæmic plague from blood. The materials are then subjected to the following examinations.

(i) **Microscopical:**—A smear is made and stained by Gram's method and with methylene blue. Leishman's stain may also be used as it gives good results. Morphological characters are then investigated and the presence of short plump bacilli, often in pairs, showing bipolar staining (Fig. 254) and Gram-negative character, is highly suggestive. It should be noted that a little before death, Past. pestis may be detected in peripheral blood by direct smear examination in about 17 per cent. of the cases.

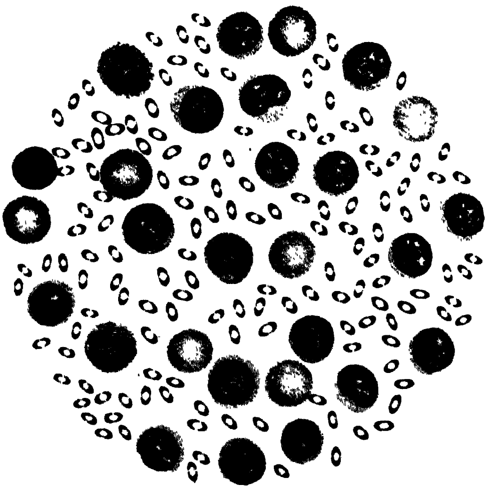


Fig. 254.

A smear examination from gland puncture showing *Pasteurella pestis* with bipolar stainin a few red blood cells and lymphocytes.

(ii) **Cultural:**—Cultures are [illegible] on agar, incubated at [illegible] to 27°C. and *not at* 37°C.

From colonies on agar plates the organism may be isolated and its cultural characters are studied in broth, ghee-broth and salt agar. Stalactite growth in ghee-broth, chain formation in broth, involution forms in salt-agar are very suggestive. Blood culture is made in the usual way.

(iii) **Animal inoculation** (Pathogenicity test):—Some of the exudate or the colonies isolated from agar may be inoculated subcutaneously into a *white rat* or *guinea-pig* to test the pathogenicity. In positive cases, the animals die in from 3 to 5 days, the organisms being present in large numbers in the lymph nodes, liver, spleen and blood. Cutaneous method, i.e., rubbing a little of the material on the shaved abdominal skin, may also be employed.

To elicit the specific action of Past. pestis with the sputum of pneumonic plague, which may contain other virulent organisms, it is best to perform the animal inoculation by applying the material to the nasal mucous membrane or to the shaved skin of the abdomen, instead of inoculating it subcutaneously.

Note:—In order to establish the identity of the organisms, pathogenicity test should always be resorted to as it forms the most reliable one.

Agglutination reaction:—During the disease, the patient's serum acquires the property of agglutinating the bacilli to a titre of 1 in 50 but this appears after the second week of the disease. The reaction is little used for diagnosis of the disease as it develops so late and as there is a natural tendency of the bacilli to cohere in clumps (auto-agglutination). It is for the latter reason also that agglutination reaction cannot be relied on for the serological identity of the organism.

The other accessory aids in the diagnosis are a very high leucocytic blood count and the haemorrhagic character of the sputum in cases of pneumonic plague (prune-juice sputum).

Pasteurella tularensis

(Bacillus tularensis; Bacterium tularense.)

The organism causes a plague-like general infection among small, wild rodents (ground squirrels and jack rabbits) in Western States of America. *McCoy* and *Chapin*, in 1912, isolated the specific organism from the blood of infected animals.

It is a small organism measuring 0·3 to 0·7 μ in length. Coccal as well as bacillary forms are encountered. It is non-motile and sometimes capsulated.

With ordinary dyes, it is not easily stained unless a mordant, such as carbolic acid or aniline, is added. Thus it can readily be stained with aniline gentian violet. It is Gram-negative.

It cannot be grown in ordinary media. Cultures, however, can be obtained in the following media, such as Dorset's egg media, blood agar and serum agar containing a piece of fresh sterile rabbit's spleen and agar containing 0·02 per cent. cystine. The colonies are small and viscid.

In animals, particularly amongst small wild rodents, the disease is very fatal and closely resembles plague. The infection is transmitted from animal to animal by blood sucking insects. On autopsy, necrotic areas are found at the site of infection and also in the internal organs, such as liver, spleen, kidneys and lungs. Mice, guinea-pigs and rabbits can be infected experimentally.

The disease is transmissible to man and may occur in the following ways:—

(i) Dealers in rabbits from handling infected animals.

(ii) Laboratory workers from handling cultures. In this respect, it resembles Malta fever.

(iii) Field workers by the bite of blood sucking insects (deer fly and tick).

In human beings, the disease is milder in nature and is rarely fatal. The infection causes an irregular fever of long duration (2 to 3 weeks), often resembling typhoid fever. Sometimes, there may be a primary ulcer at the site of inoculation with involvement of the neighbouring lymph nodes. The disease in human beings is variously named. It is called "Tularæmia" in South California, because of its greatest incidence in the neighbourhood of Tulare lake. It is also called "Ohara's disease" in Japan and "deer fly fever" in Utah.

Diagnosis of the disease consists of the following:—

(i) *Isolation of the causative organism*—The material collected for the purpose is the exudate from glands or ulcers. The organism is rarely present in the human blood. The best method of isolation is to inoculate the material into a guinea-pig from which the organism can be obtained in pure culture. The animal dies within a week and, on autopsy, the spleen and liver are found to be studded with necrotic foci. A hæmorrhagic necrosis is also noticed at the site of inoculation with corresponding glandular enlargements. Smears from the spleen show the characteristic bacilli.

(ii) *Serum diagnosis*—Specific agglutinins begin to develop in the serum of infected persons after the second week of the disease and the titre limit rises rapidly up to 1 in 1,200 or more. It is to be noted that the organism bears a serological relationship with the Brucella group, showing cross agglutination with Br. abortus (Bang).

Pasteurella pseudotuberculosis (B. pseudotuberculosis rodentium):—This organism differs from Past. pestis in the following:—

(1) Motility in 18 hours' broth culture incubated at 20° to 22°C.

(2) Alkaline reaction in litmus milk.

(3) Avirulence for white rats.

Pasteurella septica:—This group of organisms differs from Past. pestis in the following:—

(1) Fermentation of saccharose.

(2) Formation of indol.

(3) Production of a negative methyl red test.

(4) Inability to grow in the presence of bile salts, such as on MacConkey's medium.

Table showing differences between Past. pestis, Past. pseudotuberculosis and Past. septica.

	Past. pestis.	*Past. pseudotuberculosis.*	*Past. septica.*
Motility in 18 hours' broth culture at 20° to 22°C. ...	Non-motile	Motile	Non-motile
Litmus milk ...	No change	Alkaline	No change
Saccharose fermentation ...	–	–	+
Indol formation ...	–	–	+
Methyl red test ...	+	+	–
Growth in bile-salt media (MacConkey) ...	+		
Virulence for white rats ...	+	–	

+ = positive; – = negative.

CHAPTER XXXII

VIBRIO

Definition:—A vibrio is a short, curved, Gram-negative bacillus, arranged singly in the form of a comma or united at the ends, forming S—shaped curves or spirals. It possesses a single terminal flagellum and is very actively motile. A typical example is *Vibrio comma* (Vibrio choleræ) which is strictly a parasitic organism causing Asiatic cholera in man.

There are other vibrios which are widely distributed in nature, mostly as saprophytes, in water and sometimes in the fæces of man and animals and occasionally, in butter and cheese. Only a few of them are pathogenic. Besides the Vibrio comma (V. choleræ), there is another variety, *V. metchnikovi,* which produces a fatal intestinal disorder in domestic fowls with symptoms resembling those of cholera in man. In these animals, the vibrios invade the blood and tissues and may be isolated from the heart's blood of a diseased fowl.

Examples:—

A. *Pathogenic to man and animals.*

1. **Vibrio comma** *(V. choleræ)*—The causative organism of Asiatic cholera.
2. **Vibrio metchnikovi**—It causes a septicæmic disease in domestic fowls.
3. **Vibrio fœtus**—It causes infectious abortion in sheep and cattle.

B. *Potentially pathogenic.*

4. **El Tor vibrio**
5. **Paracholera vibrios**

Cause choleraic symptoms.

C. *Commensals or saprophytes (Non-p[illegible]nic to man).*

6. **Vibrio proteus** *(Finkler and Prio[illegible]io)*—Isolated from cases of cholera nostras and water.
7. **Vibrio tyrogenus** *(Deneke's vibrio* [illegible]ated from cheese.
8. **Vibrio phosphorescence**—Isolated [illegible]man fæces and water.

VIBRIO COMMA

(V. choleræ)

(Comma vibrio, Comma bacillus)

This organism was first discovered by the famous *Robert Koch,* in 1884, while working in the *Medical College Hospital in Calcutta.*

HABITAT:—It is strictly a parasitic organism, being found in the intestinal contents of cholera patients and carriers.

MORPHOLOGY:—It is a bent or slightly curved bacillus, resembling very closely a comma from which it obtained its name, "Comma bacillus" (Fig. 255). It is about $2\ \mu$ in length. The organisms may be arranged singly or two may be united at their ends, producing an S-shaped curve. When more than two are united, the form is that of a spirillum. In stained preparations, they are arranged in parallel rows, like *fish-in-stream.* This is sometimes very characteristic. It does not produce any spore nor does it possess any capsule. It has one terminal *flagellum* (monotrichate) and possesses a very active darting movement. In older cultures, there may be *involution forms.*

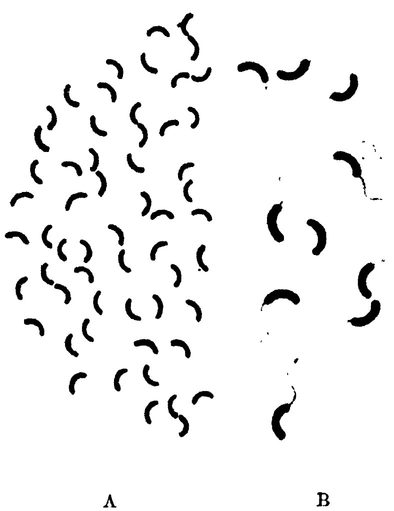


Fig. 255.
Vibrio comma, showing the comma shaped curve of the organism (A) and single terminal flagellum (B).

STAINING REACTIONS:—It is stained well with all aniline dyes and is Gram-negative. Special stains are necessary for the demonstration of the flagellum (*vide* p. 31). In practical work, a diluted solution of Ziehl-Neelsen's carbol-fuchsin (1 in 10 to 15) is a very ready and dependable staining reagent.

CULTURAL CHARACTERS:—It is aerobic and grows well in ordinary media. In *broth,* there is luxuriant growth with uniform turbidity. On *nutrient agar,* the colonies are moist, colourless and almost transparent, with overlapping.

In *gelatin stab culture,* there is a characteristic appearance. The growth is most luxuriant on the surface where there is liquefaction of the medium. In the deeper parts, there is a progressive diminution of the growth, as well as the liquefaction, producing the appearance of a funnel (Fig. 256). This is due to the fact that the organisms get less oxygen in the deeper parts than on the surface.

Special culture media:—

Dunham's peptone water (see page 69)—This is an excellent simple liquid medium for the rapid isolation of *V. comma.* The organisms usually grow on the superficial layer, forming a delicate pellicle, while the other intestinal bacteria grow in the deeper layers. Plenty of growth takes place within 6 hours. In this medium, unlike the nutrient broth, there is slight opacity but no marked turbidity.

Dieudonné's blood-alkali agar medium (see page 69)—This is a selective medium for the isolation of *Vibrio comma* from stool. The large amount of caustic potash in this medium inhibits the growth of other intestinal bacteria. The colonies are small, discrete, transparent and moist.

Taurocholate agar (p. 70):—The organism grows and produces bluish, transparent, moist colonies.

BIOCHEMICAL REACTIONS:—*V. comma* has a marked proteolytic action. This is usually observed by the *liquefaction of gelatin and coagulated blood serum.* An alkaline reaction is absolutely necessary for such a proteolysis to occur, any acid reaction inhibiting the process altogether. Rapid *production of indol* in an alkaline peptone water is another very well marked property of *V. comma.* This is usually demonstrated by a positive "cholera red" or nitroso-indol reaction. It is to be noted that nitrate present in peptone water is also reduced by the organism into nitrites. Thus the addition of sulphuric acid alone changes nitrite into nitrous acid which combines with indol, producing the nitroso-indol (red colour) reaction.

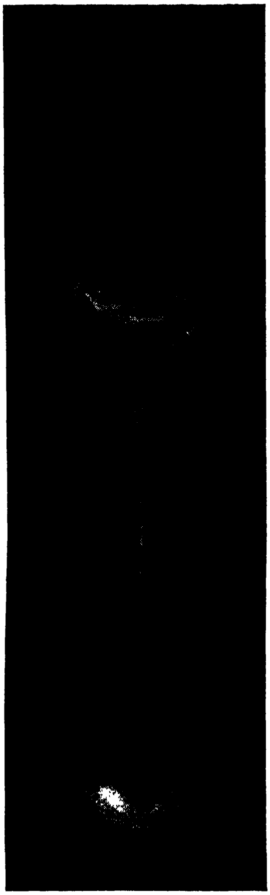


Fig. 256.
Gelatin stab-culture of V. comma, showing infundibuliform liquefaction.

Although some members of the vibrio group produce a hæmolysin which acts on the red cells causing their lysis, the Vibrio comma does not come under that category. *True cholera vibrio is non-hæmolytic.*

There is *no characteristic fermentative reaction,* although common laboratory sugars, such as glucose, mannite, maltose and saccharose, are acted on with the production of acid [illegible] no gas. It has *no action on lactose.* Usually there is no change [illegible] *milk,* but when grown for several days, the organism may [illegible] produce acid reaction followed sometimes by coagulation.

VIABILITY:—The Vibrios are all [illegible] sisting organisms, being very susceptible to heat, desiccation, [illegible] and acids. Exposure to 55°C.

kills them in less than 15 minutes, but they can remain alive at a temperature below the freezing point for some days. It is for this reason that taking of iced drinks and food stuffs is dangerous in the tropics, particularly when there is an epidemic out-break of cholera. Carbolic acid, in 0·5 per cent. solution, kills them in a few minutes. Exposure to direct sunlight and a thorough desiccation are both very injurious to the life of the Vibrios which are destroyed in a short time.

Although the organisms are so feebly resistant to desiccation, they can retain their vitality for a long period if kept moist, as in soiled clothes under conditions which prevent their complete drying. For instance, *Rogers* has recorded instances of the infection having been conveyed long distances by linen contaminated with fæcal and vomited matter from cholera patients and thus leading to out-breaks of the disease among those who had subsequently handled the clothes.

On the surfaces of fruits and vegetables, the organism may remain alive for days, specially in a cool moist place, and such articles may therefore help to spread an epidemic. The use of uncooked vegetables, as salads, and the eating of fresh fruits which have not been thoroughly washed are positively dangerous in the tropics, specially when cholera is raging in the locality.

Acids, even in low dilutions, i.e., 0·02 to 0·05 per cent. hydrochloric acid, are very injurious to the life of the vibrios which are rapidly destroyed. A healthy stomach with copious gastric juice thus affords a powerful protection against infection by *V. comma*.

The cholera organisms die out rapidly in distilled or sterile water but may survive for some time in the natural waters commonly used for drinking purposes.

TOXIN PRODUCTION:—Cholera vibrio does not produce any extracellular toxin and hence no antitoxin could be prepared. The toxic element appears to be present inside the bacterial body and is set free when the organisms die and disintegrate. This endotoxin is responsible for the toxic effects in Vibrio-infected patients.

SEROLOGICAL REACTIONS:—Although no antitoxin has been demonstrated, certain antibodies are formed in the body when it is infected with cholera vibrio. The chief of them are specific agglutinins, specific opsonins and certain bactericidal and bacteriolytic substances.

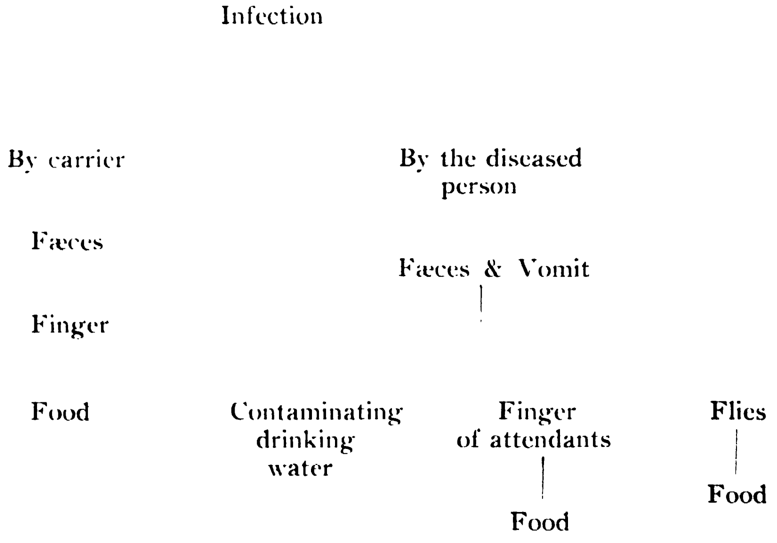
Cholera vibrio, introduced into the body of an animal, produces specific agglutinins in its blood. In this respect all the various strains of *Vibrio comma* have been found to fall into one serological type. Normally, the blood serum of a healthy man fails to agglutinate the Vibrio comma or it may do so at a low titre, i.e., in 1 in 20. But during the disease process, the [illegible] of agglutination titre may rise to 1 in 1,000 or more, although it rapidly falls after the disease is over. Carriers, harbouring the vibrios in their gall bladder or intestines, also show a high agglutination titre. In practice, a [illegible] of 1 in 100 dilution of the serum is taken as diagnostic of infection by Vibrio comma. It should be noted that, like other flagellated bacteria, *Vibrio comma* possesses two types of antigens—a heat-labile H and a heat-stable O receptor—each of which can give rise to its specific agglutinin. The immune serum produced by the injection of living or dead vibrios into rabbits contains specific bacteriolysins which can be demonstrated by the *Pfeiffer's reaction.* This test is an example of bacteriolysis *in vivo,* i.e., inside the peritoneal cavity of an immunised animal, and is employed in the identification of Vibrio comma (p. 187).

PATHOGENICITY

IN ANIMALS:—Animals do not naturally suffer from the infection, but under experimental conditions, when living vibrios are injected into the peritoneal cavities of rabbits or guinea-pigs, the animals die of toxæmia within 24 to 48 hours. As a rule, V. comma is not pathogenic to pigeons.

IN MAN:—Vibrio comma produces the disease known as Asiatic cholera. The main seat of the lesion is the alimentary canal, particularly the small intestine. The vibrios usually enter the system by the mouth through contaminated food and drinks. Like typhoid fever, there is usually a direct or indirect contact either with a patient suffering from the disease or a carrier. In the tropics, an additional source of danger is the fly which feeds on the dejecta of cholera patients and subsequently contaminates articles of diet by carrying the vibrios both internally as well as externally.

The following table summarises the mode of infection in cholera:—



In the majority of cases, the acid gas[illegible] e kills the organisms inside the stomach, but if the bacilli can pass [illegible] ned into the alkaline duodenal content, they multiply with astounding [illegible] ty and produce the signs and symptoms of the disease. Lowering of [illegible] eral resistance, faulty digestive function of the stomach, constipation [illegible] etetic errors which upset the digestion, always favour infection.

Lesions

Intestines:—In the small intestine, there is marked vascular engorgement, catarrhal inflammation with desquamation of the epithelium and exudation usually of a serous, but occasionally sero-sanguinous, fluid, mixed with flakes of mucus, into the lumen of the gut. Such an evacuation constitutes the socalled "rice water stool" of cholera. The toxins of *V. comma* act injuriously on the liver cells, causing a suppression of the biliary secretion and it is for this reason that the cholera stool is not coloured with bile. The stool is passed frequently through the rectum, constituting the typical cholera evacuations. There is also frequent vomiting of similar material. Besides the vascular engorgement and the changes in the mucous membrane of the gut, there is a very marked hyperplasia of the lymphoid tissues of the intestines. The Peyer's patches, as well as the solitary follicles in the submucous tissue of the intestine, are greatly swollen and enlarged and they stand out very prominently, looking like small tubercles on the epithelial surface. This is a very characteristic appearance in the small intestine of a case of cholera. The peritoneal surface of the intestine shows marked vascularity and appears injected (Fig. 257).

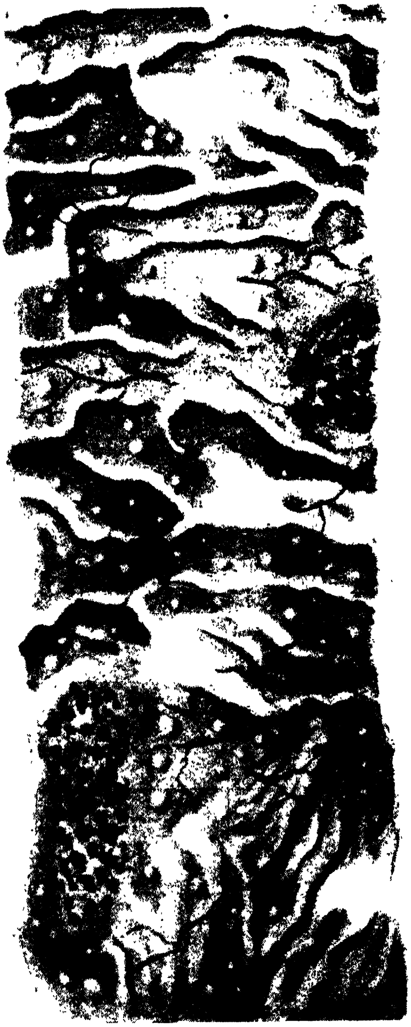


Fig. 257.

Cholera intestine.

Note the prominence of lymphoid follicles and Peyer's patches, injection of blood vessels and denudation of surface epithelium with flakes of mucous shreds.

The *mesenteric lymph nodes* are always considerably enlarged and hyperæmic, with marked hyperplasia of the lymphoid tissue elements.

The vibrios may be seen to penetrate up to the submucous layer but no further and even this is found only in fatal cases, possibly as a terminal phenomenon. The organisms *never* penetrate further or *reach the general blood stream.*

Gall bladder:—Another organ which becomes the seat of an important lesion is the gall bladder. The vibrios appear to reach the organ from the duodenum, via the biliary passage, and cause an acute or chronic catarrhal inflammation of the mucous layer. The epithelial cells are swollen and desquamated and there is production of an excess of mucus which is discharged into the bile. The vibrios multiply rapidly in the bile which becomes almost a pure culture of the organisms. They are discharged with the bile into the duodenum to be passed with the fæces and such a gall bladder may thus serve as a reservoir of infection. Usually, the discharge of the organisms from an infected gall bladder into the intestine is intermittent, and a person with such a gall bladder acts as a carrier. Calculi may form in such an infected organ.

Kidneys:—The toxins of the *Vibrio comma* are very injurious to the kidneys which are often damaged more or less seriously according to the intensity of the toxæmia. The organs are swollen and enlarged; their capsules are stretched and they are pinkish red in colour. On section, the cut surfaces are vividly pink with injected vessels passing towards the cortex. The pyramids appear intensely red and almost hæmorrhagic, specially at their bases (Fig. 258). Microscopical examination reveals marked engorgement of the glomerular and intertubular vessels. The glomeruli are swollen with some exudation of an albuminous fluid into the capsular spaces while the tubular epithelium shows varying degrees of fatty, granular and hyaline degeneration. In some instances, the lumina of the tubules are found to be completely blocked by a coagulated material which thus appears to cause an obstruction to the flow of urine. The results of such an alteration in the kidneys produce suppression of urine and signs and symptoms of acute uræmia which is always a very serious complication in cholera. It may be noted in this connection that the above mentioned changes in the kidneys suggest more a picture of acute nephrosis rather than one of acute nephritis. From a careful study of the histological appearances of the kidneys in a large number of cholera cases, the writers are of opinion that, contrary to the current belief, uræmia in cholera is not due to acute nephritic changes in the kidneys [illegible] as occur in scarlet fever and other streptococcal infections. The [illegible] appears to be the result of

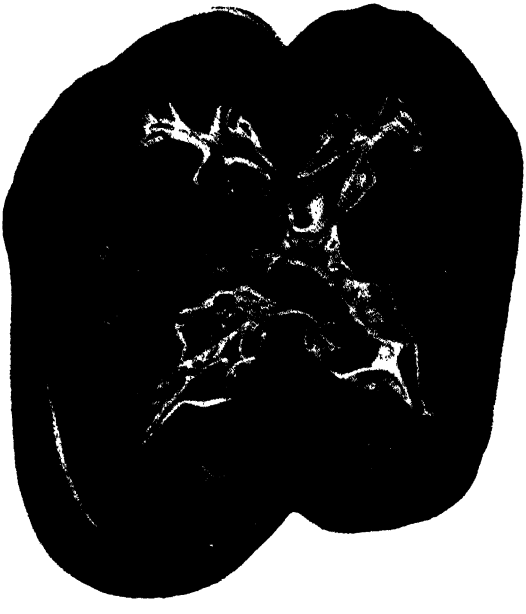


Fig. 258.

Cholera kidney.

Note the intense congestion of pyramids and the presence of injected blood vessels in the cortex. Histopathology of such a kidney did not show any nephritic change.

an overwhelming toxæmia, producing a functional paralysis of the organs, aided by a loss of tone of the vasomotor system, particularly of the splanchnic regions. Such a toxic nephrosis lasts only for a short time and the kidneys usually recover completely both in function and structure during convalescence.

Changes in the blood:—Owing to depletion of fluid through vomiting and purging, the blood becomes concentrated and its specific gravity high, rising above 1060. For the same reason, the number of red and white cells per cubic millimetre also rises very considerably. There is considerable diminution in the amount of chlorides and also in the alkali reserve, the latter being responsible for a condition of some degree of acidosis. The blood pressure always falls below normal.

Carriers

Persons, while convalescing from an acute attack of the infection, harbour the vibrios in their gall bladder and intestines and discharge them with the stools. They act as carriers and are the most important elements in the spread of the disease. Although the percentage of such carriers is very large, fortunately the condition does not last long. Usually, the excretion of vibrios by convalescents continues only for two to three weeks and it is extremely unusual to detect the organisms in the stool two months after recovery from the disease. Thus cholera carriers are of less importance epdiemiologically as compared with typhoid carriers where the condition lasts longer.

During an epidemic outbreak, large numbers of healthy persons who come in contact with the cases may be found to excrete the vibrios in their stools and such persons are called healthy or contact carriers and their incidence may rise as high as 6 to 7 per cent. during the epidemic period. They are very important elements in the dissemination of the disease, although the actual period during which they harbour the vibrios in their intestines is very short, lasting for 6 to 8 days only.

THERAPEUTIC CONSIDERATIONS

Although the specific antiserum contains agglutinins, bacteriolysins and other immune substances and the injections of such sera protect animals which are experimentally infected, their use in actual cases of Asiatic cholera in man has been found to be totally disappointing. The disease also runs too acute a course to be affected by any vaccine therapy.

Bacteriophage in Cholera:—

It has been definitely found out that a very powerful lytic agent is present in the stool of a convalescent case of cholera. This "phage" is said to kill the vibrios within a few hours. Owing to its powerful lytic effects, the phage is now-a-days extensively used as an active therapeutic agent in the treatment of cholera as well as in its prophylaxis in epidemic areas. It has been suggested that the presence of this phage in the intestines of cholera patients during the period of convalescence is responsible for the disappearance of the vibrios from the stool in such a short time and the excretion of this powerful agent, while the patient is convalescing, into tanks, streams and other sources of drinking water has also been supposed to be an important factor in the control of an epidemic in rural areas.

The discovery of the cholera bacteriophage has led to its use in the treatment of cholera cases and reports have been made from different parts of India about its efficacy in this disease. It is essential that for this purpose, the disease must be detected early and the phage administered at the earliest moment. It is supplied in ampoules and should be given in empty stomach in sufficient doses and should be repeated frequently. Of course, the accessory methods of medical treatment for urgent symptoms should be resorted to in addition to the bacteriophage therapy.

Mode of administration:—"It should be given undiluted in one drachm doses every 30 minutes. Two bottles, each containing 50 c.c., should be finished by direct sipping in the first 16 hours. After the first 24 hours, one bottle during the next 48 hours should generally suffice (Harvey)."

Cholera vaccine:—This was first introduced as a prophylactic inoculation against cholera by Haffkine who used living organisms for this purpose. The vaccination consisted of a preliminary injection of an attenuated culture, followed later by that of organisms of exalted virulence. As it is not desirable to employ a vaccine consisting of highly virulent living organisms, the use of a vaccine containing dead organisms has recently been recommended. It consists in giving two subcutaneous injections of an emulsion made from virulent, highly agglutinating cholera vibrios and killed by heat at 55°C. The first dose is 4,000 millions to be followed 7 to 10 days later by the second dose consisting of 8,000 millions. Some local and general reactions are produced but they pass off in 24 to 48 hours.

Such an inoculation is associated with the development of specific agglutinins and other antibodies in the blood of the inoculated person and is supposed to confer some degree of protection against the vibrio infection. This artificial immunity, however, does not last for more than six months and is most active during the first 2 or 3 months.

Bilivaccine:—This has been introduced on the hypothesis of Besredka that real protection can only be obtained if the local cells in the intestinal epithelium can be stimulated to heightened resistance against the penetration of the cholera toxin. To produce such an action, vaccination by the mouth is now frequently resorted to in mass immunisation. The vaccine is administered either as a suspension or as tablets and is preceded by a dose of bile. This preliminary administration of bile is supposed to increase the subsequent action of the vaccine. Both the bile and the vaccine should be given on empty stomach. Usually 3 to 5 doses are enough.

It has been found by some that a solid local immunity is thus produced without the appearance of demonstrable antibodies in the blood and this method of immunisation is reported to [illegible] diminished the incidence of cholera cases in endemic and epidemic [illegible]

LABORATORY DIAGNOSIS OF CHOLERA

Collection of materials:—

From infected patient:—Rice water stool, vomit and other fomites and blood.

From a dead body:—Fomites, such as soiled linen, contents of the small intestine and bile from the gall bladder. A coil of intestine should be ligated at both ends and then removed and sent to the laboratory for examination.

From suspicious water:—About one litre should be collected from the surface in a sterilised vessel and packed in ice until used for examination.

From carriers:—Fæces, bile from duodenum and blood.

Methods of examination

Microscopic examination of the fresh stool:—This gives an idea of the character of the cellular exudate and is very helpful to differentiate the condition from acute bacillary dysentery. There are very few leucocytes or macrophages but only mucus and degenerated epithelial cells. In some cases, red cells may be present. Many bacilli with characteristic morphology and well marked motility may be seen (Fig. 259).

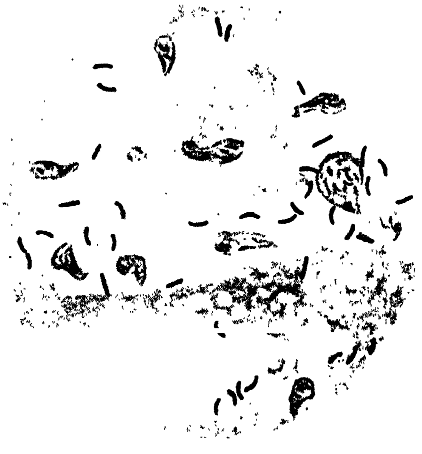


Fig. 259.
Cholera stool.

A white flake of mucus is taken and smeared over a clean slide which is allowed to dry in the air. The film is then fixed over the flame and stained with dilute carbol fuchsin (1 in 15 of Ziehl-Neelsen's stain) for a minute or two. After a thorough wash, when the slide is dry, it is examined under an oil immersion lens. The typical *comma shaped vibrios* may be found. They are Gram-negative.

Cultural methods:—An alkaline peptone water tube (Dunham's) is inoculated with a flake of mucus and incubated for 8 to 10 hours. A drop from the surface should be examined for the vibrios.

Sub-cultures may then be made from the surface material on agar, fresh peptone tube and special media and the details of the morphology and biochemical reactions studied.

The two media which are particularly useful are (a) taurocholate agar (pH. 8·6 to 9·6) and (b) blood-alkali-agar (Dieudonne's medium). Plates of the above media may also be inoculated with a flake of mucus thoroughly washed in sterile saline. The characteristic colonies will be found after 24 hours.

Identification of the cultivated organisms may be made by studying the following details:—

- *(i) Morphology.*
- *(ii) Biochemical reactions:—*
    - *(a) Liquefaction of gelatin.*
    - *(b) Cholera-red reaction.*
    - *(c) Hæmolysis.*
- *(iii) Agglutination in vitro with specific antiserum.*
- *(iv) Pfeiffer's reaction—Agglutination and bacteriolysis in vivo (p. 187).*

For diagnostic criterion of cholera vibrio, vide table on page 527.

Blood examination:—This should be done for three reasons:—

1. To eliminate algid type of malaria in the tropics.
2. To demonstrate the presence of specific agglutinins in the serum.
3. To test the specific gravity which is always raised very high in cholera. This also serves as an indicator as to how much saline should be injected.

In a very acutely fatal case of cholera, no agglutinins are found. But when the patient survives for a few days or during convalescence, specific agglutinins are present in considerable amount. It has been found that the maximum titre is reached at the end of the first week after which it begins to decline. In practice, the presence of agglutinins in the serum in a dilution of 1 in 100 and above is quite diagnostic. In "carriers," there remain sufficiently demonstrable agglutinins in the blood to establish a diagnosis of Vibrio infection.

Sanitary considerations:—It may be remembered in this connection that the two important factors in the infection by *V. comma* are drinking water and food materials contaminated by flies. Hence, every precaution should be taken against them. All water used for drinking purposes should always be boiled and any other material, such as dishes, utensils, etc., which may be used in connection with eating and drinking should be thoroughly sterilised either by boiling or by suitable antiseptics. Use of chlorine and potassium permanganate in the sterilisation of water has been found to be very efficacious. All food materials should be cooked fresh and taken immediately after and all precautions should be taken to protect them from contamination by flies. Milk is a particular source of danger and it should never be permitted to be taken without sufficient boiling each time it is taken. To prevent insanitary conditions which always attract flies, liberal use of bleaching powder, quick lime or any other suitable disinfectant should be insisted on.

When an epidemic breaks out in [illegible]ity, tanks, wells and similar sources of drinking water should be at [illegible] protected from contamination, But usually in a country where people are ignorant, these sources are already contaminated before any step can be taken and it is for this reason that sterilisation of water is so important.

The question of carriers and human reservoirs of cholera vibrios is always a very important matter. They should be detected as early as possible and isolated. Such men should not be allowed to handle food or drink or to come in contact with the latter in any way.

Personal prophylaxis:—The general health should be maintained as satisfactorily as possible. Fatigue, overwork, mental worry, panic and apprehension, night-keeping and in fact, excess of any kind, should be avoided. The diet should be plain, simple and easily digestible. Dietetic indiscretion tends to upset digestion and increase susceptibility to infection. Raw fruits, hard indigestible foods, salads, melons, cucumbers and such other articles of diet should be avoided during an epidemic of cholera as they tend to produce indigestion and catarrh and increase the liability to infection. Purgatives should be avoided for the same reasons. Apart from the various precautions mentioned previously under sanitary conditions regarding food and drinks, weak tea or lemon decoctions are very good prophylactics. The regular and frequent use of acids and acidulated drinks are very good during an epidemic as the acids are very injurious to the life of the cholera vibrio and 10 to 20 drops of sulphuric or hydrochloric acid in a glass of water taken two or three times a day are very effective.

ALLIED ORGANISMS

An organism (*El Tor vibrio*) was isolated by Gotschlich (1906) from patients suffering from dysenteric symptoms at El Tor, which was found to produce marked extracellular toxin and having hæmolytic action. Serum reactions, however, conform to V. comma and it has been regarded by some to be a variation in the type of V. comma and the cases were possibly cholera carriers.

It has been further observed that there occurs a group of cases with choleraic symptoms which are of less severity than true cholera. The vibrios isolated from these cases do not conform to the classical V. comma but differ in being markedly hæmolytic, not agglutinated by anti-cholera serum and giving a negative Pfeiffer's reaction. These organisms are referred to as *Paracholera vibrios*.

Other vibrios

Vibrio metchnikovi (*Gameleia*, 1888):—This organism causes a septicæmic disease with acute enteritis in fowls and was isolated from their fæces and blood. (Note:—Chicken cholera is produced by Past. avisepticа). In its morphology, staining reactions and some of the cultural characters, it resembles V. comma but differs from the latter in the following:—

(i) Gelatin is liquefied rapidly.

(ii) It is hæmolytic.

(iii) It is not subjected to lysis or agglutinated by anti-cholera serum.

(iv) It is more virulent to guinea-pigs and pigeons.

(v) This organism is more invasive than V. comma, being recovered from the blood of infected and inoculated animals.

Vibrio fœtus (*Macfadyen and Stockman,* 1913, *Smith,* 1918):—This organism causes infectious abortion in sheep and cattle. Morphologically and in staining reactions, it resembles cholera vibrio but for its growth in artificial media it requires a micro-ærophilic condition. It does not produce indol. Serologically, it is a distinct and homogeneous species, being agglutinated by its specific antiserum. Laboratory animals are not susceptible.

Vibrio proteus (*Finkler and Prior's vibrio,* 1884):—This organism was isolated from cases of "cholera nostras." It has frequently been found in water. It resembles cholera vibrio morphologically and culturally and it can be grown in ordinary media. It liquefies gelatin rapidly and does not give any cholera-red reaction. It is not agglutinated by anti-cholera serum. It is less virulent than Deneke's Vibrio tyrogenus.

Vibrio tyrogenus (*Deneke's vibrio,* 1885):—This organism was isolated from cheese. It liquefies gelatin but not so rapidly as V. proteus. It does not give any cholera-red reaction and is not agglutinated by anti-cholera serum. It is pathogenic to guinea-pigs and pigeons.

Vibrio phosphorescence:—This organism has been isolated from human fæces and has frequently been encountered in water.

Table showing the differences between various organisms of the Vibrio group:—

Organisms.	*Gelatin liquefaction.*	*Hæmolytic properties.*	*Cholera-red reaction.*	*Agglutination with anticholera serum.*	*Pfeiffer's reaction.*	*Pathogenicity for laboratory animals.*
V. comma	Slight			+	+	+; Scarcely pathogenic to pigeons.
V. metchnikovi ...	Marked			o	o	+; highly pathogenic to pigeons.
V. fœtus ...	Marked	+		o	o	o
Vibrio proteus ...	Very marked	+		o	o	+
Vibrio tyrogenus	Marked	+		o	o	+
Paracholera vibrio	Slight	+		o	o	+

Spirillum minus (*Carter*, 1887)

(Spirochæta morsus muris)

The organism of rat-bite fever.

This spirillum occurs naturally in rats (Fig. 260) and other wild rodents. It is a small spiral organism, measuring 2 to 5 μ in length. It possesses a number of terminal flagella at each pole (lophotrichate) and is an actively motile organism (Fig. 261). It can be stained readily with Leishman's stain. For cultivation, Smith-Noguchi's medium should be used. The organism is pathogenic to guinea-pigs. In man, it produces a disease characterised by a relapsing type of fever, associated with a local lesion at the site of inoculation, involvement of the corresponding lymph nodes and sometimes an eruption of macular rash. The disease is usually developed about 10 to 20 days after the bite of a rat. The easiest way to diagnose the disease is either by actually finding the organism in the peripheral blood, which appears to be seldom successful or injecting the blood into a mouse in which it does not produce any lethal effect but the organism can be demonstrated in the animal's blood. The organism may also be demonstrated from the local lesion and the corresponding lymph glands. It is to be noted that one intravenous injection of neosalvarsan or other arsenical preparations effects a complete cure.

Fig. 260.

Rattus rattus, the carrier of the organism of rat-bite fever (Spirillum minus).

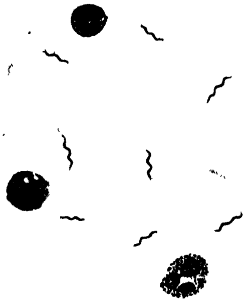


Fig. 261.

Spirillum minus in the blood plasma of rat, showing two or three curves with flagella at each end. The actual number of spirilla is, however, fewer than that shown in the diagram.

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CHAPTER XXXIII

SPIROCHÆTES

It is "a general term for all elongated, motile, flexible organisms which are twisted spirally around their long axes."

Systemic position:—The features which bring them closely in relation to bacteria are the following:—

(i) Absence of any definite localised nucleus.

(ii) There are no sexual phenomena of reproduction and they multiply like bacteria by transverse division.

The features which differentiate them from bacteria are the following:—

(i) Some of them possess an undulating membrane like trypanosomes.

(ii) They possess motility without flagella.

(iii) They are flexible.

(iv) Some of them like the protozoa, pass stages of their life cycle in intermediate hosts, such as the louse and tick.

(v) They undergo lysis when treated with certain chemicals, such as saponin and sodium taurocholate, as opposed to the resistance shown by most bacteria.

Differentiation of spirochætes from spirillæ:— Spirillæ are bacteria with curved bodies and possessing motility and definite flagella. They are not flexible like the spirochætes.

Classification:—According to their *morphological characters*, the organisms are divided into 6 different classes (Fig. 262) as follows:—

(1) **Spirochæta** (Ehrenberg, 1834) or Coiled hair (*Spira*, a coil; *Chæta*, hair).

Characters:—An axial fibre is present. Body is twisted around this axial fibre in a spiral manner, like a staircase. Secondary waves may be superimposed on the primary spirals during motion. Metachromatic granules are present in their bodies.

Examples:—These are free living fresh water or marine species of which *Spirochæta plicatilis* is the type.

(2) **Saprospira** (Gross 1911) or Putrid coil (*Sapros*, rotten

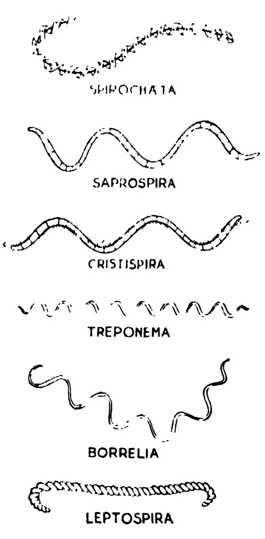

Fig. 262.

Diagram illustrating the morphological characters of various forms of spirochætes.

Characters:—There is no axial filament and no crista (undulating membrane) or flagellum. Body is divided by many transverse septa into chambers.

Examples:—These are large free swimming aquatic organisms.

(3) **Cristispira** (Gross, 1910) or crested coil (*Crista*, a crest; *Spira*, coil).

Characters:—It possesses a band like membrane or crista (undulating membrane) which runs in a spiral manner along the length of the organism. Body is divided into chambers by septa of thickened cytoplasm.

Examples:—These exist as parasites in the alimentary tracts of oysters and other shell fish.

Type—Cristispira balbianii.

(4) **Treponema** (Schaudinn, 1905) or turned thread.

Characters:—There is no axial fibre and no crista or undulating membrane. It possesses primary spirals, which may be either closely or loosely wound. The ends may be thin or filamentous. During active movement, the coils do not tend to open out and the organisms maintain rigid cork-screw like spirals.

Examples—

Treponema pallidum.
Treponema perteneue.
Treponema microdentium.
Treponema macrodentium.
Treponema retringens.
Treponema gracile.
Treponema balantidis.

(5) **Borrelia** (Swellengrebel, 1907) or **Spironema.**

Characters:—These are like treponema but possess flexible and snake-like curves which tend to straighten out. These organisms are disintegrated by a 10% solution of saponin and bile salts.

Examples:—

Organisms of relapsing fever (Borr. obermeieri, Borr. duttoni, etc.)
Borrelia anserina.
Borrelia theileri.
Borrelia gallinarum.
Borrelia vincenti.
Borrelia buccalis.
Borrelia bronchialis.
Borrelia curvgyrata.

(4) **Leptospira** (Noguchi, 1917) or fine coil (*Leptos*, thin or small).

Characters:—There is no axial fibre and no crista. Cytoplasm is not chambered. Many closely wound, primary spirals. Their ends are turned round at a sharp angle to the rest of the body. Hence, when the organisms remain at rest, the ends appear characteristically hooked. They resist the action of 10% saponin but are dissolved by bile salts.

Type species—Leptospira icterohæmorrhagiæ.

Motility:—The organisms do not possess any flagella but are still actively motile. Three kinds of movements can be observed:—

(1) Flexion or bending of the filament, producing a change in shape.

(2) Rotation around the long axis.

(3) Movement of translation, i.e., moving from one place to another. They can move either forwards or backwards without exhibiting any anteroposterior polarity. The rate of movement is extremely variable. Some move slowly while others dart quickly across the field of the microscope.

Staining reactions:—Spirochætes are very difficult to stain with ordinary aniline dyes and this is due to the want of nucleo-proteins in their bodies. Direct films from an exudate may be stained with (a) Giemsa's stain (long exposure is necessary, *vide* p. 33.) or by (b) Fontana's method (silver staining). These organisms, when stained by Gram's method, are all *Gram-negative*. Sections of tissues may be stained by Levaditi's method (vide appendix).

Dark ground illumination

This method is of great value for the examination of spirochætes and other delicate organisms in the living condition as they are not visible when unstained by the ordinary microscope. In this, the rays of light are thrown on the object very obliquely so that none of them enters the tube of the microscope directly and reaches the examining eye. They can only reach the observer's eye when they are scattered by objects, e.g., bacteria, which are of different refractive index from the medium in which they are suspended. The objects are thus rendered visible as bright points of light in a dark background. In order to achieve this, the following three requisites are necessary:—

1. A special form of condenser.

2. A very bright illuminant, such as an electric arc or 'pontolite' lamp.

3. A special mount for the 1/12 in. oil immersion objective, containing a funnel stop which reduces the numerical aperture of the objective to less than 1·0. Or else to use an objective with a built-in-diaphragm, in order to cut off all direct rays of light.

Examination of the specimen:—Slides employed for the purpose should be 1·0 to 1·1 mm. thick. Film from the exudate is made as thin as possible and covered with No. 1 coverslip. It is advisable to smear the rims of the coverslip with vaseline so as to prevent evaporation. A drop of oil is placed on the undersurface of the slide as well as on the top lens of the condenser. The slide preparation is then placed on the stage and contact between the two drops of oil is established. Using the plane side of the mirror, the light is directed into the dark ground condenser and the preparation is focussed with a low power objective (2/3 in.). A concentric ring of light is obtained, which, if not in the centre of the field, is brought into position by manipulating the two centring screws of the condenser. A drop of oil is now placed on the top of the coverslip and the oil-immersion lens is adjusted. The specimen is then carefully focussed. A slight adjustment of the mirror or the condenser may be necessary to obtain a uniformly dark background with brightly illuminated bacteria and other organisms in the field.

After use, the parts of the microscope should be wiped clean to remove the oil.

Fontana's method of staining spirochætes

Reagents required:—

1. Fixative

Glacial acetic acid	1 c.c.
Formalin	20 c.c.
Distilled water	100 c.c.

2. Mordant

Tannic acid	5 gms.
Carbolic acid	1 gm.
Distilled water	100 c.c.

3. Staining solution

This is an ammoniacal silver nitrate solution which is prepared by adding 10 per cent. ammonia solution drop by drop to a 0·25 per cent. silver nitrate solution in distilled water until the precipitate formed just dissolves. Now, more silver nitrate solution is added drop by drop, until the liquid becomes just opalescent.

Technique:—

(i) Dried films are fixed without heat in solution No. 1 and stained for about a minute. The fluid is dropped on to the film and changed once or twice.

(ii) The preparation is then well washed in running water.

(iii) The film is then covered with the mordant and heated (without boiling) till steam rises and allowed to remain for about half a minute.

(iv) The slide is again washed in water.

(v) Ammoniacal silver nitrate solution is poured over the film and heat is applied gently till steam rises and then allowed to remain for half a minute.

(vi) The film is finally washed well in distilled water, dried, *mounted in Canada balsam* and examined with an oil immersion lens.

By this method, the spirochætes are coated with a deposit of silver which makes them stand out very prominently as black filaments which, however, are much thicker than the living organisms.

Cultivation:—Essential conditions required for growth are the following:—

(i) Native animal protein, such as blood, serum or ascitic fluid.

(ii) Low oxygen pressure (anaerobic condition)—This is obtained by adding to the medium a piece of fresh sterile kidney or testis of a rabbit.

(iii) Covering with a layer of paraffin oil—This helps in preventing evaporation of moisture and loss of CO_2.

(iv) Liquid or semi-solid medium.

(v) Slightly alkaline reaction of the medium.

(vi) Sugars are essential for the metabolic activity of blood spirochætes, specially for their movements.

It is to be noted in this connection that while some grow very easily, such as the *Leptospira ictero-hæmorrhagiæ*, others do not, such as the *Treponemata*. Hence, the latter are generally preserved by animal inoculation.

According to their cultural requirements, they can be divided into two main groups as follows:—

1. Aerobic—This includes the Leptospira only.

2. Anaerobic group—This includes tissue and blood spirochætes, such as Treponema and Borrelia groups and also the saprophytic spirochætes.

Medium for the cultivation of Leptospira

Composition:—

Rabbit serum	2 parts.
Saline or Ringer's[1] solution ...	6 parts.
Citrated rabbit plasma[2]	1 part.
Nutrient agar (2 per cent.) at 60°C. ...	1 part.

The contents are mixed by rotation. A layer of sterile liquid paraffin, ½in. deep, is then placed on the surface of the medium.

1. *Ringer's solution*

Sodium chloride	9 gms.
Calcium chloride	0.25 gm.
Potassium chloride	0.42 gm.
Distilled water	1,000 c.c.

2. *Citrated rabbit plasma*—For this, 10 c.c. of blood are mixed with 1 c.c. of 10 per cent. sterile sodium citrate solution, centrifuged and the clear supernatant plasma is pipetted off.

Media for anaerobic group

Smith-Noguchi's fluid medium (p. 383) is used for this purpose. When the spirochætes have to be cultivated from impure or contaminated materials, a solid medium is to be preferred. It is prepared by adding two parts of 2 per cent. nutrient agar at 50°C. to one part of sterile ascitic fluid. About 15 c.c. of this mixture are poured into a long narrow test tube, containing a piece of sterile rabbit's kidney or testicle at the bottom. The tube is then allowed to cool and the mixture solidifies. Inoculation of the material containing the spirochætes is carried out by means of *stab inoculation* with a platinum loop or a capillary pipette. The medium is then covered with a thick layer (half an inch) of sterilised paraffin oil or vaseline. The requisite anaerobic conditions are produced by the tissue and entrance of oxygen is prevented by the column of solid agar and liquid paraffin or vaseline.

Once pure cultures have been o[illegible]d, subcultures may be made in Smith-Noguchi's fluid medium w[illegible] exactly the same as above, excepting the agar mixture. Such a [illegible]n may also be employed when cultivation is made from pure materia[illegible] should be noted that it is better to introduce the inoculating material along with the sterile animal tissue before adding the ascitic fluid. After inoculation the tubes are placed inside an anaerobic jar as an additional safeguard.

Resistance:—Power of resistance is not very great outside the animal body. Although they cannot stand desiccation, the organisms may remain alive if kept in the cold for a long time. All pathogenic varieties are rapidly destroyed when exposed to a temperature of 50°C. and above. Ordinary sunlight has little or no effect on the spirochætes.

Toxin production:—The toxins of spirochætes have not yet been identified and it is very difficult to explain the way in which pathogenic spirochætes act on the host.

Serological reactions:—Spirochætes behave very similarly to bacteria in their serological reactions. When a person suffers from a disease or when the spirochætes are injected into animals, they give rise to the production of agglutinins, spirochætocidins, spirochætolysins, complement fixing and other immune bodies.

Adhesion phenomenon (*Brown* and *Davis*, 1927):—The principle of the reaction lies in the fact that under the influence of the specific antisera, the spirochætes may be altered in such a way as to allow minute particles and bacteria to adhere to them in clumps. No such clumping and adhesion take place in the presence of non-specific serum.

Pathogenicity:—The organisms can be divided according to their habitat and pathogenic properties into the following:—

I. Those not found in man and animals

1. *Spirochæta.*
2. *Saprospira.*
3. *Cristispira.*

II. Those found in man and animals

4. *Treponema.*
5. *Borrelia.*
6. *Leptospira.*

The latter may again be divided into two main groups—*saprophytes and pathogens.*

A. Saprophytes or commensals

Mouth—*Borrelia buccalis.*
Teeth and gums—*Treponema microdentium*
Treponema macrodentium.
Respiratory tract—*Borrelia bronchialis.*
Intestinal canal—*Borrelia eurygyratum.*
Genital area—*Treponema refringens.*
Treponema balantidis.

B. Pathogens.

1. Blood spirochætes.
   (a) *Of man*—Organisms of relapsing fever (*Borrelia obermeieri, Borrelia duttoni,* etc.)

(b) *Of animals*—

Borrelia anserinum (geese), *Borrelia theileri* (cattle), *Borrelia gallinarum* (fowls), *Borrelia muris* (mice).

2. Tissue spirochætes.

Treponema pallidum (Syphilis).
Treponema pertenеue (Yaws).
Borrelia vincenti (Vincent's angina).

3. Blood and tissues.

Leptospira icterohæmorrhagiæ (Weil's disease).
Leptospira hebdomadis (Seven day fever of the East).

Pathogenic spirochætes of man can also be divided according to their mode of transmission:—

(a) Transmitted by direct contact.

Treponema pallidum.

Treponema pertenеue.

(b) Transmitted by intermediaries.

(a) Blood sucking insects.

Louse—*Borrelia obermeieri.*

Tick—*Borrelia duttoni.*

(b) Rats—*Leptospira icterohæmorrhagiæ.*

(c) Field mice—*Leptospira hebdomadis.*

(c) Transmission unknown—*Borrelia vincenti.*

Therapeutic considerations:—It will be seen later that spirochætal infection is followed by the development of an active immunity. But the inoculation of such *anti-spirochætal sera* offers very little protection. The only spirochætal infection where serum therapy is of any value is Weil's disease. *Vaccination* with dead spirochætes has also been tried in various animal spirochætoses but the results are not very satisfactory.

The treatment of various spirochætoses is based on chemotherapy and the following heavy metals are used for the purpose—mercury, bismuth, arsenic, antimony and gold. The salts of these drugs which are employed, have a marked parasitotropic but least organotropic action. These chemotherapeutic agents do not act direc[illegible] the spirochætes but possibly through the medium of the natural defens[illegible]chanism of the body (reticulo-endothelial system). It is to be noted th[illegible] of the spirochætoses, except leptospira infection (Weil's disease), [illegible]d very well to arsenical preparations. Appearance of arsenic [illegible] strain should be borne in mind while treating spirochætal diseases [illegible]ularly relapsing fever.

TREPONEMA PALLIDUM

(Spirochæta pallida)

The causative organism of syphilis.

Schaudinn and **Hofmann,** 1905.

HABITAT:—The organism is found in large numbers in syphilitic lesions and has a strictly parasitic existence.

MORPHOLOGY:—*Treponema pallidum* is an exceedingly delicate spiral organism, measuring about 6 to 14 μ by 0·25 μ. It is characterised by sharp deep regular curves, the number of which on an average is ten. Each curve is situated at a distance of 1 μ, the depth of the curve being 1 to 1·5 μ. The ends are pointed and tapering, terminating at each end into a fine thread. It does not possess any flagellum. The organism possesses a low refractivity and, when viewed under dark ground illumination, appears as silvery white. The morphology of the organism is well demonstrated in the fresh state when it shows active movements but at rest it appears as dots and dashes. The following types of movements may be observed:—(1) rotary cork-screw like motility along its long axis, (2) a flexion of the whole body and (3) a to-and-fro gliding movement by alternate contraction and expansion of its coils; the latter, however, remain relatively rigid. The organism, though actively motile in its place, shows very little power of locomotion and progression. This is a very important distinguishing point in identifying the organisms from other saprophytes which may be encountered in the syphilitic lesions. These latter, on the other hand, move very rapidly from one place to another.

STAINING REACTIONS:—*Treponema pallidum* is very difficult to stain, the ordinary aniline dyes being without any effect. It is for this reason called *pallidum*. For the staining of spirochætes, *vide page* 531.

CULTIVATION:—*Treponema pallidum* is an anaerobe and grows best at 30°C. It does not grow in ordinary media. The organism can, however, be cultivated in Smith-Noguchi's solid medium (agar—2 parts, ascitic fluid—1 part and a piece of sterile rabbit's kidney or testicle). The medium is inoculated with the exudate by means of a capillary pipette (stab inoculation) and incubated at 37°C. for 2 to 3 weeks. Treponema and other bacteria grow along the line of inoculation but later, i.e., after about 10 to 14 days, the Treponema wanders away from the stab culture and spreads in the medium as a hazy diffuse growth. A pure culture can be obtained from the edge of this growth by cutting the tubes, fishing out the colonies and transplanting into another serum-agar-tissue tube. Smith-Noguchi's fluid medium is used when cultivation is made from pure cultures.

For the maintenance of cultures, the solid medium is preferable but it has been found that the virulence of Treponema pallidum is soon lost by such artificial sub-cultures. Hence, the best way to preserve these organisms is by intratesticular inoculation into a rabbit (*vide infra*).

RESISTANCE:—*Treponema pallidum* is a very delicate organism and cannot withstand desiccation, being rapidly destroyed by drying. When exposed to moist heat at 51°C., it is killed within an hour. Cold is, however, well borne by the organism.

SEROLOGICAL REACTIONS:—Infection with *Treponema pallidum* produces complement fixing bodies which give a positive Wassermann reaction. It also leads to the production of precipitins which characterise various types of flocculation reaction, such as Kahn's test, Sachs-Georgi, Sigma and Meinicke's reactions. Both the complement fixation test and flocculation reactions are of great value in the diagnosis of syphilis and its various manifestations. *Wassermann reaction is not a specific complement fixation test* as meant by Bordet-Gengou reaction (p. 186). It should be noted that the antigen used in the Wassermann reaction is not specific, as lipoidal extracts of normal organs may give this reaction. Antibody developed in the syphilitic infection is therefore a lipoidophilic substance which is naturally present in the serum in minimal amounts and is augmented non-specifically. This lipoidophilic antibody is supposed to be contained in the globulin fractions of the serum proteins.

ANIMAL INOCULATION:—Majority of the animals appear to be refractory to experimental inoculation with *Treponema pallidum.* It is only in anthropoid apes (Chimpanzee) that a disease comparable to syphilis can be produced. The organism is introduced by scarifying the skin of the areas of eyebrows or genitals. It has been observed that subcutaneous, intravenous and intraperitoneal inoculations appear to be ineffective. In this animal, primary and secondary stages of the disease can be produced but not the tertiary stage, because the animal dies of broncho-pneumonia before it has time to develop tertiary lesions.

Of all the laboratory animals, the *rabbit* is the only one which is, to a certain extent, susceptible to the infection when introduced into certain sites. Thus, inoculation into the anterior chamber causes iritis, scarification of the cornea leads to keratitis and intratesticular injection produces orchitis in which large numbers of the organisms may be found. It is to be noted that in this animal the disease produced is usually a localised one.

When the organism is inoculated into a *mouse,* it does not produce any lesion but treponemata may be recovered from its lymph glands, spleen and brain, several months afterwards.

Rabbit syphilis:—This is a naturally occurring chronic disease found in the neighbourhood of the mouth and [illegible]ia of rabbits and is caused by *Treponema cuniculi.* Microscopically [illegible] is indistinguishable from Tr. pallidum. The infection can be tr[illegible]d experimentally only to rabbits but not to any other animals. I[illegible]bbit, the organisms do not penetrate deeper and have little tendency [illegible]ad to the internal organs.

PATHOGENICITY

Treponema pallidum causes a chronic, specific, contagious, venereal disease, *syphilis*, marked by various kinds of lesions. The course of an acquired disease is usually described under the following stages:—

Primary syphilis, characterised by a chancre (sore at the site of inoculation) and indolent buboes.

Secondary syphilis, characterised by eruptions on the skin and mucous membranes, sore throat and general enlargement of the lymphatic glands.

Tertiary syphilis, characterised by gummata in various internal organs (visceral syphilis) and severe skin lesions.

Quarternary syphilis or *parasyphilis,* characterised by nervous manifestations, such as tabes dorsalis and general paralysis of the insane.

Each of these periods is separated by a period of remission or a quiescent interval in which the symptoms disappear and the lesions heal up spontaneously to reappear again in another guise.

(*Note:*—The word syphilis is borrowed from the name of a shepherd, called "*syphilus,*" from the latin poem of Fracastoro, published in 1530.)

Mode of infection:—

(i) The disease is generally contracted by sexual intercourse and is particularly a venereal affection. The organism is directly introduced into the area and gains entrance into the body through some abrasions in the skin or mucous membrane. After an incubation period which may vary from 3 to 6 weeks, a papular sore appears at the site of inoculation. This is known as the *chancre (genital).*

(ii) Such chancres may also develop in other parts of the body, such as lips, fingers, breast, etc., having no connection with sexual intercourse. Hence they are called *extra-genital chancres.* The infection in these cases is obtained from contamination with syphilitic exudate as through kissing, the use of infected utensils, towels, etc., or through shaving with a contaminated razor. Such cases are of frequent occurrence amongst nurses, doctors, dentists and other medical attendants. As already mentioned, the organism can hardly survive for a long time outside the body and for this reason the occurrence of extra-genital chancres is not so common.

(iii) The disease can also be *transmitted congenitally* through the placenta, particularly by a syphilitic mother. The infection of the fœtus usually occurs *in the second half of pregnancy.* It will be noted that there is no primary stage in congenital syphilis and both the lesions of secondary and tertiary stages are mixed up.

After inoculation, the organisms not only multiply locally but are also carried to the nearest lymph node by the lymphatic current, within a very short time (in less than an hour). Thus it will be seen that during the early period of the infection, the spirochætes rapidly get into the blood and tissues without, however, producing any clinical signs of the disease.

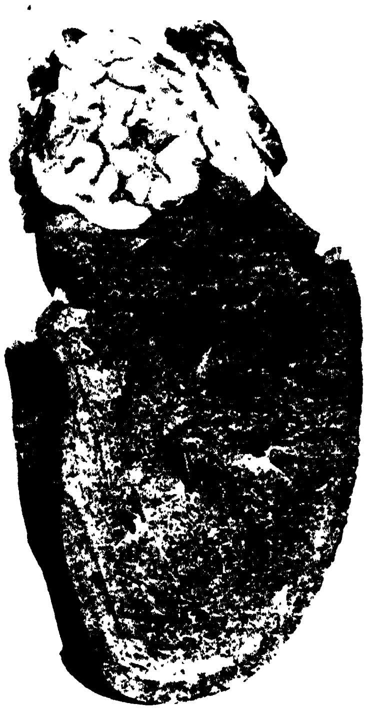

Fig. 263.
Gumma liver.

Histopathology:—Whatever may be the stage of the disease, the histological lesion produced is essentially the same. The characteristic change is the formation of an infective granuloma, composed of a cellular exudate which consists mainly of plasma cells and lymphocytes. This is often associated with obliterative endarteritis in the vessels around the parasitic focus. The subsequent course of the lesion will depend on the stage of the disease in which it is produced. It may lead to coagulative necrosis or fibrosis but both the processes are often combined. The necrotic lesion in tertiary syphilis is of the consistency of gum, and hence it is called a *gumma* (Fig. 263). As syphilis is a chronic disease, the ultimate effect is the formation of fibrous tissue which by its contraction may lead to scarring and crippling of the organ.

THERAPEUTIC CONSIDERATIONS

The remedies employed for the treatment of syphilis are the preparations of arsenic, mercury and bismuth all of which are spirochæticidal. Iodides are also used to help the above drugs by removing the necrotic mass and thereby exposing the parasites to their action.

Malaria therapy in general paralysis of the insane (Wagner—Jauregg, 1919):—This is one of the fever or pyrogenetic therapies commonly employed in cases of G.P.I. and some cases of neurosyphilis where Wassermann reaction is persistently positive. The method consists in inoculating benign tertian malarial parasites (*Plasmodium vivax*). Usually 10 paroxysms are allowed to occur after which they are stopped with quinine.

Syphilis and marriage

Marriage is prohibited in syphilitics who are capable of transmitting the disease. But it may be permitted to men by whom the disease is not transmissible. The latter cannot be insured definitely unless there is a definite standard of cure. If a person remains absolutely free from all symptoms for a period of two years following an intensive treatment carried over for a period of 2 to 3 years, with several negative Wassermann reactions, he may then be relatively certain of cure. It has been stated that *five years must elapse after infection* before the person may be allowed to marry.

LABORATORY DIAGNOSIS OF SYPHILIS

This consists of the following:—

1. *Demonstration of the causative organism by—*
   - (a) *Microscopical examination*
     - (i) *Dark ground illumination of a fresh specimen.*
     - (ii) *Staining of a smear by Giemsa's or Fontana's method.*
     - (iii) *Staining of Treponema in tissues by Levaditi's method.*
   - (b) *Cultivation*
     - (i) *In vitro—Noguchi's medium.*
     - (ii) *In vivo—Inoculation into the testis of rabbits.*
2. *Serum diagnosis*
   - (a) *Wassermann reaction.*
   - (b) *Flocculation test—Khan's test, Sachs-Georgi's reaction, Sigma reaction, Meinicke's reaction.*
3. *Examination of the cerebrospinal fluid in cases of neuro-syphilis.*
4. *Luetin reaction—An allergic phenomenon.*

Demonstration of the causative organism:—This is only successful from the primary sore (genital or extra-genital) and enlarged lymph nodes during the primary stage as well as from the cutaneous rashes and mucous patches during the secondary stage. It is very difficult or rather impossible to demonstrate the Treponemata in the lesions of tertiary syphilis as they are very scanty. They have, however, been found in the peripheral parts of gummata in neurosyphilis and cardio-vascular syphilis.

Collection of materials:—

From the primary sore—When collecting materials, no antisyphilitic remedies should be applied to the sore. The purulent material is washed with normal saline. The edge of the sore may be gently rubbed with a sterile gauze or gently scraped with a blunt scalpel so as to denude the surface epithelium, thereby promoting the flow of clear serum. Such a specimen is supposed to contain a considerable number of the organism. Material collected in this way eliminates the surface organisms which may be confused with Treponema pallidum. It should be noted that the saprophytic spirochætes are invariably superficial. If blood exudes, it should be wiped away and the clear serum is taken for examination. The presence of blood should be avoided as far as possible, otherwise it obscures the Treponemata.

The clear serum may be drawn out with a capillary tube, the opening of which is held horizontally in the secretion as it oozes out. The tube fills by means of capillary attraction. The ends of the tube are sealed by melted paraffin. Such tubes may be sent to a distant laboratory and living treponemata can be demonstrated even after 14 days or more.

From enlarged lymph node—Usually the gland is punctured with a sterile hypodermic needle and a little juice is aspirated for examination.

Nature of examination:—The best result is obtained by examining the organism in the living condition under a dark ground illumination and this provides a convenient means of rapid diagnosis. The *Treponema pallidum* is recognised by the following characters:—

(i) Silvery white appearance.
(ii) Extreme thinness.
(iii) Sharp and regular coils, seven of which are equal to the diameter of a red cell.
(iv) Active motility in its own place without any locomotion.

Failing dark ground illumination, staining methods may be resorted to. When stained by Giemsa's method, *Treponema pallidum* is coloured a faint pink whereas other saprophytic organisms are stained deep blue.

Saprophytic organisms which may be present in syphilitic lesions and thus cause confusion are the following:—

(a) *In the genital area*
  (i) *Treponema refringens.*
  (ii) „ *gracile.*
(b) *In the mouth*
  (iv) *Treponema microdentium.*
  (v) „ *macrodentium.*

These organisms can easily be excluded by proper collection of materials. *They differ from Treponema pallidum* in the following respects:—

1. They appear glistening and not silvery white under dark ground illumination.

2. They are coarse, highly refractile organisms with open spirals.

3. They are actively motile organisms, moving rapidly across the field.

4. They are stained easily and take a deep blue stain with Giemsa's stain.

Serum diagnosis:—This is of no value in the diagnosis of early primary stage. The various serum reactions, such as Wassermann and Kahn's flocculation tests, become *positi[illegible]bout two weeks after the appearance of the primary sore* and becom[illegible] marked during the secondary stage and remain positive throughout [illegible]se of the disease. They may, however, diminish during remissions [illegible] anti-syphilitic treatment. It should be noted that while treating a [illegible] Kahn's test remains positive longer than the Wassermann reaction i[illegible] of the cases. As already mentioned, the Wassermann reaction thoug[illegible]le is not truly specific as it is positive in other diseases, such as yaws, leprosy, sleeping sickness, glanders, malaria and acute miliary tuberculosis. In these cases, Kahn's test may be of value.

In cases where the clinical evidence is suspicious and the serum reactions are negative, a '*provocative injection*' of 0·45 gm. of N.A.B. or other arsenical preparations may be given and the test carried out one week later. By this means a definitely positive result is sometimes obtained.

Examination of the cerebrospinal fluid:—The various examinations that are carried out are cytological count, globulin test, Lange's colloidal gold test, Wassermann reaction and Kahn's test. The findings in a case of neurosyphilis are shown in a tabular form as compared with a normal case.

	Neurosyphilis.	*Normal case.*
Cytological count	Lymphocytosis (10 to 50 cells or more per c. mm.)	1 5 cells per c. mm.
Globulin test ...		Negative.
Colloidal gold test ...		Negative.
Wassermann and Kahn's tests		Negative.

Luetin reaction:—Noguchi observed that like tuberculosis a certain amount of hypersensitiveness exists in syphilis. Intradermal injection of an extract of culture of Treponema pallidum causes an allergic response in the syphilitic person, particularly in the secondary and tertiary stages. The positive result is indicated by the formation of a papule or pustule. This test is not used for diagnostic purposes owing to the presence of other more reliable tests.

In ***conclusion,*** the diagnosis of syphilitic infections may be summed up according to the stage of the disease as follows:—

I. **Congenital syphilis:**—Blood of the mother or the child is examined for Wassermann reaction. In the case of the still born fœtus, the causative organism (Treponema pallidum) can be demonstrated in large numbers in the liver, spleen (Fig. 264) and kidneys by Levaditi's method of staining.

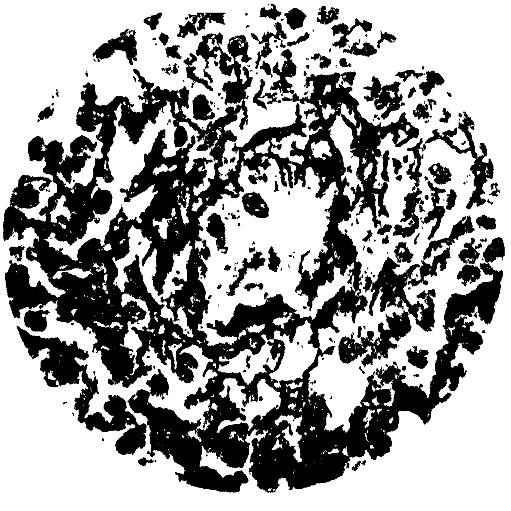

Fig. 264.

Section of a spleen from a case of congenital syphilis stained by Levaditi's method, showing a large number of *Treponema pallidum* distributed round an arteriole.

II. **Acquired syphilis.**

1. *Primary stage.*

(i) Demonstration of Treponema pallidum from the primary sore and enlarged lymph node by dark ground illumination or staining of smears by Giemsa's or Fontana's method.

(ii) Therapeutic test—Disappearance of the primary sore with the administration of mercury and arsenical preparations.

NOTE:—Serum diagnosis is of no value in the early stage of the disease.

2. *Secondary stage.*
   - (i) Demonstration of Treponema pallidum from cutaneous rashes and mucous patches.
   - (ii) Markedly positive Wassermann and flocculation tests.
   - (iii) Therapeutic test—Disappearance of rashes with the administration of arsenical and mercurial preparations.

3. *Tertiary stage.*
   - (i) Positive Wassermann, Kahn's and other flocculation tests. In doubtful cases, provocative methods should be employed.
   - (ii) Therapeutic test—Amelioration of symptoms by anti-syphilitic drugs.

NOTE:—Demonstration of the organism from the exudate is not easy and convenient.

4. *Quarternary stage* or stage of neuro-syphilis.
   - (i) A positive Wassermann and Kahn test in the blood.
   - (ii) Changes in the cerebrospinal fluid *(vide supra)*.

NOTE:—Demonstration of the organism is not possible in life.

TREPONEMA PERTENEUE

(Castellani, 1905)

The causative organism of yaws.

Treponema perteneue is morphologically indistinguishable from Tr. pallidum. In human beings, it causes a highly contagious disease known as yaws or framboesia tropica. It is essentially a disease of the tropics, affecting the dark-skinned races. Like syphilis, it also consists of three stages but the primary lesion is extragenital and the secondary stage is characterised by fever and peculiar raspberry-like papules. Tertiary lesions may follow later but viscera and central nervous system are never involved. Yaws is neither congenital nor venereal in origin. The infection is obtained by direct contact and the organisms gain entrance through cuts, abraded skin or insect bites. Children are specially liable to be attacked. Patients suffering from the disease give a positive Wassermann reaction. The disease is amenable to treatment with arsenical preparations and one injection may suffice to ensure a complete cure. It has been said that the patient suffering from yaws is protected against syphilis and it has also been regarded by some as a modified form of syphilis. It should be noted that, in the skin lesions, the organisms are found in the superficial layer of the epidermis and not in the corium, as in syphilis and proliferation of the endothelium and perivascular round cell infiltrations are absent.

BORRELIA OBERMEIERI

(Spironema recurrentis)

The spirochaete of relapsing fever.

The organism was discovered by **Obermeier** in the Berlin epidemic of 1867-68 in the blood of patients suffering from relapsing fever during the febrile paroxysm. The observation was, however, published in 1873.

HABITAT:—It is a strictly parasitic organism.

MORPHOLOGY (Fig. 265):—The organism is spiral-shaped and the undulations or curves are not closely woven. The coils are more or less open and their number varies according to the length of the organism, which varies from 10 to 30 μ. The thickness is about 0·35 to 0·5 μ. When seen in the fresh state, the organism is actively motile, possessing three kinds of movement as described before. It shows a cork-screw like motility, lateral oscillation and progression in either direction. During such movements, the curves tend to straighten out. In stained preparations, the organism appears homogeneous, without any definite cellular structure. Sometimes, granules (chromatin dots) may be visible at places. It has tapering pointed ends, without any flagella. If the blood is examined towards the end of the paroxysm, the organisms may be seen to be aggregated in rosette forms, due to the action of the developing antibodies. The organisms multiply by transverse fission. They are flexible and, owing to this, they can pass through filters which retain the ordinary bacteria.

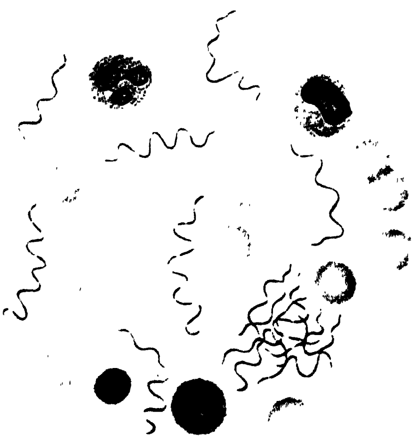

Fig. 265.

Blood film from a case of European relapsing fever, showing a large number of *Borrelia obermeieri*. At one place, the organisms can be seen to be arranged in a rosette form.

STAINING REACTIONS:—Vide page 531.

CULTIVATION:—This organism, though not strictly anaerobic, yet prefers to grow best in reduced oxygen tension. For a successful cultivation, about 2 to 5 c.c. of peripheral blood should be taken aseptically during a febrile paroxysm and inoculated into Smith-Noguchi's fluid medium (vide p. 533). Subculture is to be made between the fourth and seventh days.

SEROLOGICAL REACTIONS:—It has been said that one attack of the disease confers immunity. Antibodies of the nature of agglutinins and lysins appear in the blood, which cause clumping and disintegration of the organisms. Some of them are phagocytosed by macrophages and this chiefly takes place in the spleen. Inspite of all these destructive influences, some remain latent in the brain and other tissues. Later, when the circulating antibodies have decreased, they multiply and cause the first relapse. These resisting forms evoke another output of antibodies which cause a disappearance of the organisms, resulting in crisis and elimination of the latter from the peripheral blood. These antibodies, however, are specific but inactive against a subsequent relapse strain.

Other organisms which are associated with relapsing fever differ in their serological characters and are therefore regarded as different species.

ANIMAL INOCULATION:—The susceptible animals are monkeys, white mice and white rats but not rabbits or guinea-pigs.

PATHOGENICITY:—It produces the disease known as relapsing fever, characterised by an incubation period (2 to 10 days), a pyrexial period and a period of remission and relapse. Each pyrexial period lasts for about 5 to 7 days and is followed by a crisis. The organisms are found in large numbers in the peripheral blood just before the crisis but in the interval, they retire to the internal organs, such as the brain, spleen and the liver. After about a week, a relapse occurs but is of much less severity and of shorter duration. In this way about 3 or 4 relapses occur. The disease is not very fatal, having a mortality of 2 to 5 per cent.

Mode of infection:—The disease is transmitted by the blood sucking insect (Fig. 266), *Pediculus corporis* (body louse). The lice become infective 16 days after biting a patient suffering from relapsing fever. It is probable that during this time, the spirochætes undergo some sort of developmental changes in the stomach of the body louse. Twenty-four hours after an infected feed, the organisms can be demonstrated inside the stomach but after this time they disappear. In about 8 to 10 days' time they reappear in the body fluids and are distributed all over the body, including the legs and antennæ. When once infected, they remain so for the rest of their lives and they even transmit the infection to the second generation. The infection is conveyed not by the bite of the louse but by the contamination of the wound with the excreta of the insect or its contents when the latter is crushed by the victim during scratching.

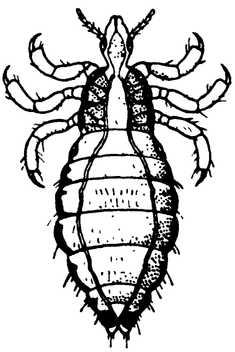

Fig. 266.
Pediculus corporis
(body louse).

Other louse-borne relapsing fevers are:—

(i) *Indian relapsing fever—Borrelia carteri.*

(ii) *North American relapsing fever—Borrelia novyi.*

(iii) *North African relapsing fever Borrelia berberum.*

THERAPEUTIC CONSIDERATIONS:—No serum or vaccine has been prepared to be of value in relapsing fever. The injection of arsenical preparations, however, often prevents relapses and cures the disease.

LABORATORY DIAGNOSIS

This consists in demonstrating the organism in the peripheral blood. For this purpose, the latter should be taken during the height of the fever and it should be subjected to the following examinations:—

(a) Dark ground illumination.

(b) Staining by Leishman's, Giemsa's or Fontana's method—A very thin film should be made for the purpose.

(c) Culture in Noguchi's medium—This is not usually employed.

(d) Inoculation into rats or mice—About 2 to 5 c.c. of blood are injected intraperitoneally into a mouse or rat. In the mouse, the organism appears in the peripheral blood within 24 hours and persists for 3 to 4 days and then disappears. These organisms may appear during relapses (3 or 4 may follow each other) but their numbers are very few. In the rat, the organism appears within 40 hours and disappears after 2 days. Relapses are not seen in rats. Infected animal survives a long time without any fatal result. Spirochaetes of relapsing fever may be demonstrated either by a microscopical examination of the blood taken from an infected mouse or rat or by cultivating the citrated blood in Smith-Noguchi's fluid medium.

Borrelia *(Spironema)* ***duttoni***

The causative organism of African relapsing fever (Tick fever)

Dutton and **Todd,** 1905.

Morphologically and *biologically,* this organism resembles Borrelia obermeieri but differs from the latter in the following:—

1. It is transmitted by a tick, *Ornithodorus moubata* (Fig. 267). The infection is obtained not by biting but by the contamination of the wound with the infective excreta and the secretion of the coxal glands of the insect. The insect remains infective for a long period after a meal from an infected host and the infectivity may be transmitted to the second generation by the female insect.

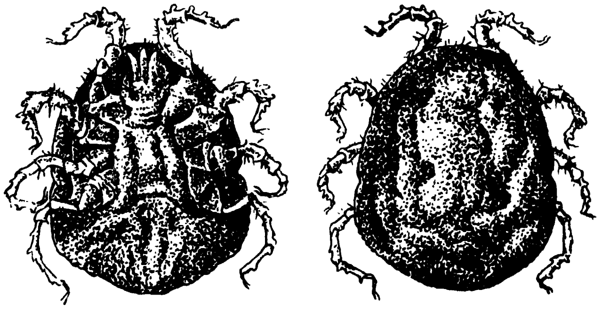

Fig. 267.
Ornithodorus moubata (Tick).

2. The disease produced by Borr. duttoni is characterised by short periods of pyrexia (2 or 3 days), many relapses and very low mortality.

3. It is pathogenic to monkeys and various laboratory animals, such as guinea-pig, mouse and rat.

BORRELIA VINCENTI

(Spironema vincenti)

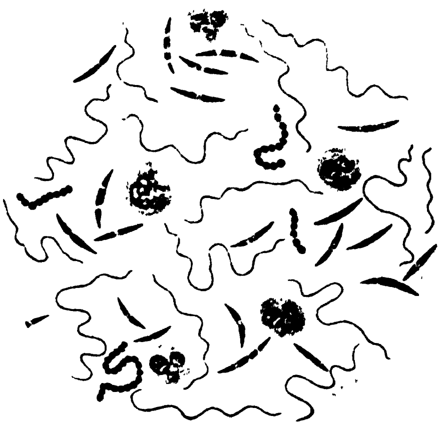

Fig. 268.

A smear of throat swab from a case of Vincent's angina, showing the spirochætes and fusiform bacilli.

This spirochæte is a long delicate organism with open irregular curves. It is often found in association with a Gram-negative bacillus (Fig. 268), *Fusiformis dentium* (B. fusiformis) in the throat of patients suffering from the disease known as Vincent's angina. The latter is characterised by the formation of a false membrane, followed later on by ulceration. Fusiformis dentium measures about 3 to 10 μ in length, with tapering ends. The organism is usually straight or curved. It stains readily with ordinary aniline dyes, but unequally showing a beaded appearance. Both the organisms can be cultivated anaerobically.

LEPTOSPIRA ICTEROHÆMORRHAGIÆ

The causative organism of infectious jaundice (Weil's disease)

Inado and **Ido**, 1914.

HABITAT:—It is a parasitic organism of rats and field mice.

MORPHOLOGY:—The organism measures about 6 to 9 μ in length and 0·25 μ in thickness. It possesses numerous, perfectly regular, closely wound coils which are so fine that they are not demonstrable in the stained preparations except under dark ground illumination. In addition to these primary coils, secondary curves may also be seen. The characteristic morphological feature of the organism is that one or both ends are sharply curved forming terminal hooks, giving the whole organism a resemblance to the letter C, S, *b* or *l*. This is of great diagnostic importance, differentiating it from other spirochætes. The movement is similar to that of other spirochætes.

STAINING REACTION:—Vide page 531.

CULTIVATION:—The organism is [illegible] and grows best at 25° to 30°C. The optimum pH is about 7·6. T[illegible]ritional requirement is simple and the organism can thrive in distill[illegible] with a little addition of potassium nitrate. The medium used [illegible] growth of leptospira is described on page 533. Subcultures sh[illegible] made every 4 to 6 weeks.

RESISTANCE:—The *L. icterohæmorrhagiæ* is very sensitive to acids and is rapidly destroyed by bile. In wet, damp soil, they can remain alive for months.

SEROLOGICAL REACTION:—Protective antibodies, of the nature of agglutinins and lysins, appear in the blood of patients suffering from the disease by the tenth day. These can be demonstrated by guinea-pig inoculation (*vide infra*). Complement fixing bodies are also present in the immune sera, which may be utilised in the diagnosis of convalescent cases.

PATHOGENICITY:—The organism produces an acute febrile illness in which the patient becomes jaundiced on the fourth or fifth day of the disease. In severe cases, there is a tendency to hæmorrhages from the various mucous membranes and the skin. The course of the disease can be described as follows:—

Incubation period—This is usually about 5 to 7 days.

Febrile stage—This lasts for a period of 6 or 7 days and is characterised by high fever, muscular pains, congested eyes and albuminuria.

Icteric stage—This lasts for a period of 6 days and is characterised by progressive, deepening jaundice with hæmorrhages. Death may take place during this stage.

Convalescent stage—There is a gradual subsidence of jaundice and other symptoms, with the development of various protective antibodies in the blood.

Mode of infection:—The organism commonly occurs in wild rats (Fig. 269) and field mice which act as carriers of the disease. In these animals, *Leptospira icterohæmorrhagiæ* are present in the kidneys (Fig. 270) and are excreted in the urine, contaminating food and water. Infection in human beings takes place chiefly through intact or abraded skin, particularly in the damp soil in coal mines which harbour the leptospira. The organism may also gain entrance through the nasal mucosa or conjunctiva from the water contaminated with rat's urine. Evidences are on record where laboratory workers were infected

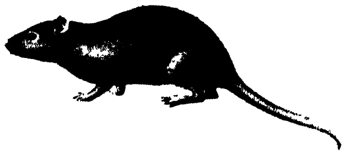

Fig. 269.

Wild rat, the reservoir of the organism of Weil's disease.

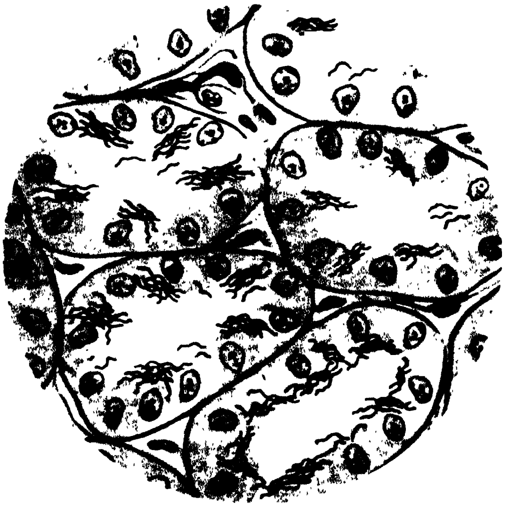

Fig. 270.

Leptospira icterohæmorrhagiæ inside the tubules of the kidney of a wild rat.

in the above ways from the infected guinea-pig's material. Infection through the alimentary canal is not possible as the organisms are destroyed by the acid gastric juice and the bile in the intestine. Thus, the disease is primarily one of the rodents and man is secondarily infected. Infection from man may occur but is relatively rare.

THERAPEUTIC CONSIDERATION:—Injection of antisera obtained from horses often reduces the mortality of the disease. It is given in doses of 60 c.c. subcutaneously on the first day and then repeated in doses of 20 c.c. for 2 or 3 days. In bad cases, the injection may be given intravenously. It should be noted that Weil's disease is the only spirochætal infection which is not amenable to arsenical treatment.

Prophylaxis:—This consists of proper and efficient drainage and exclusion of rats.

DIAGNOSIS

This consists of the following:—

1. *Demonstration of the causative organism.*
2. *Demonstration of the protective antibodies in the serum.*

Demonstration of the organism:—During the first week of the disease, the organisms are distributed throughout the body and can be demonstrated in the blood. After the seventh or ninth day, they disappear from the blood and appear in the urine, reaching the maximum during the third or fourth week. After the fifth week they can rarely be found in the urine. Thus, for a bacteriological diagnosis, the blood should be examined during the first week, after which the urine. The organism is detected by the following methods:—

(1) Microscopical examination, particularly by the dark field method.

(2) Cultural examination.

(3) Guinea-pig inoculation.

Blood:—The best way to demonstrate the organism in the blood is to inject 3 to 5 c.c. of citrated blood *intraperitoneally* into a *guinea-pig*. This leads to an infection of the animal which usually dies in about 7 to 12 days. The symptoms are fever, jaundice and hæmorrhages. Post mortem, there is intense jaundice in the subcutaneous tissues and hæmorrhages throughout the body. The hæmorrhagic areas in the lungs occur as small and large spots, resembling the 'wing of a butterfly.' This is often described as characteristic. Spleen is enlarged and congested. The organisms are present in large numbers in the blood and internal organs. By Levaditi's method of staining, *Leptospira icterohæmorrhagiæ* can be demonstrated in considerable numbers in the liver where they are situated in the interstitial tissues surrounding the liver cells. Instead of waiting for the death of the animal, it may be killed on the fourth day when there is a marked rise of temperature and a smear examination of blood will show the leptospira.

Cultural examination may be carried out by inoculating the leptospira medium (p. 533) with 2 to 3 c.c. of bl[illegible]. The organisms are searched by the dark field method weekly, at least for 4 weeks. Direct microscopical examination fails to detect the organisms as they are rarely present in sufficient numbers.

Urine:—The leptospira is best demonstrated by examining the centrifugalised deposit by the dark field method. The morphology of the organism is much altered, showing a typical and degenerated forms. Injection into a guinea-pig is often unsuccessful and the results are atypical as the organism loses its virulence due to the presence of acid and bile in the urine.

Demonstration of protective antibodies

1. *By protection experiments*:—Injection of the patient's serum and leptospira into the guinea-pig prevents the death of the animal, whereas in the control, the animal dies of leptospira infection.

2. *By the phenomenon of bacteriolysis in vivo*:—This is based on the same lines as Pfeiffer's phenomenon in the case of cholera vibrio. Patient's serum and the leptospira are injected into the peritoneal cavity of a guinea-pig and lysis of the organisms is observed under the microscope.

Reickenbergh reaction *(Thrombocytobarin test; Adhesion phenomenon)*:—This reaction is employed for the identification of the species of leptospira. When a fluid culture of the *Leptospira icterohæmorrhagiæ* is mixed with its specific antiserum (patient's serum) and is allowed to be acted on for some time (20 minutes), the organisms undergo some alterations which make them adhere to particles, such as blood platelets and bacteria. This phenomenon can be observed under the microscope.

LEPTOSPIRA HEBDOMADIS

Ido, Ito and **Wani,** 1918.

This organism is the cause of seven day fever in the East and is morphologically indistinguishable from Leptospira icterohæmorrhagiæ but it differs serologically from other leptospiras. It is pathogenic to guinea-pigs. A species of field mouse is found to be the reservoir of the organism in whose urine it is excreted. The mode of infection is similar to that of Leptospira icterohæmorrhagiæ. The organism can be demonstrated in the peripheral blood of patients suffering from the disease; it also appears in the urine in the later stages of the disease.

CHAPTER XXXIV

FILTRABLE VIRUSES

Definition:—It has been already mentioned that there are certain infective agents which are beyond the range of visibility of the ordinary microscope and their presence can only be demonstrated by the effect they produce when inoculated into susceptible animals. These agents, owing to their minute size, are capable of passing through a porcelain filter and are called *filtrable viruses.*

Types of bacterial filters:—These include the following:—

1. **Berkefeld filter**—It has been made into three grades of porosity—V (veil) the coarsest, W (wenig) the finest and N (normal) the intermediate.

2. **Chamberland filter**—This has been graded into various sizes and numbered 1 to 13.

3. **Seitz filter**—In this, the filter surface consists of a film of asbestos. This is made into 3 grades—coarse, normal and special E. K. The disc is renewed after each filtration and the old one is discarded.

4. **Collodion filter**—This is a membrane filter whose porosity depends on the percentage of the solution employed. It has been graded into various sizes and is now-a-days used for measuring the size of the viruses. It has the advantage of overcoming the fallacies regarding the adsorption phenomenon which takes place in the above filters.

Thus it will be seen that all the filters can be broadly divided into the two following groups:—

(a) *Coarse filters* which are used only as clarifying apparatus.

(b) *Fine filters* which are employed to obtain a bacteria-free filtrate as in the preparation of bacterial toxins and also for sterilisation of sera in making media.

Methods of filtration:—The easiest way in which filtration may be accomplished is by suction. The fluid to be filtered is placed on the outside of the candle and the suction applied by means of an exhaust pump as shown in the Figure 271. The negative pressure may be estimated by attaching a mercury manometer. For [illegible] purposes, a negative pressure of 200 to 300 mm. of mercury is sufficient.

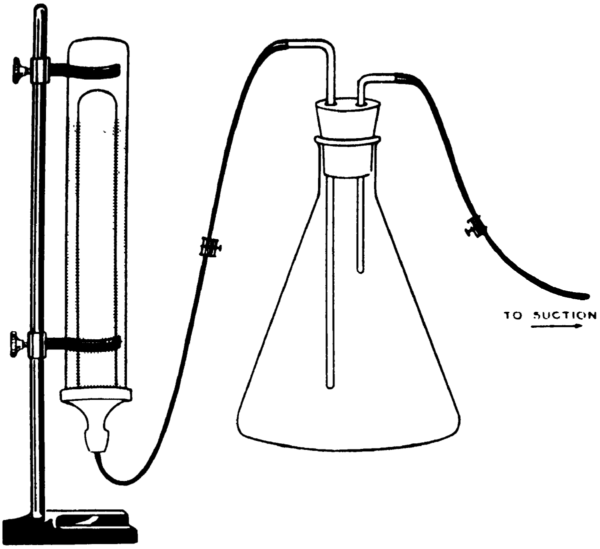

Fig. 271.
Method of filtering through a Berkefeld filter.

Before using the filter candles, they should be tested for flaws and leaks. For this purpose, a test organism, such as *Serratia marcescens* (B. prodigiosus), is included in the fluid to be filtered and the filtrate is then cultivated, both aerobically and anaerobically, to see whether there is any growth. If no growth is detected, the filter candle is perfect. These candles after use should be cleaned in distilled water with a stiff nail brush and then boiled in distilled water. They are finally sterilised in the autoclave and kept ready for future use.

Factors influencing filtration:—It should be noted that the failure of a virus to pass through a filter does not necessarily mean that its particles are too large. The filtration is a physico-chemical phenomenon and the factors which influence the process are the following:—

(i) The type and porosity of a filter.

(ii) The plasticity of the organism.

(iii) The amount of negative pressure employed.

(iv) The duration of filtration.

(v) The temperature at which the filtration is carried out.

(vi) The amount of protein and other substances present in the solution.

(vii) Electrical charge of the virus and the material composing the filter.

(viii) Reaction of the fluid in which the organisms are suspended.

Examples of filtrable viruses:—

Those producing disease in man

Smallpox (variola) and cowpox (vaccinia).

Chickenpox (varicella).

Herpes febrilis and herpes zoster.

Molluscum contagiosum.

Infective warts.

Rabies (common also to dogs).

Epidemic poliomyelitis (Acute anterior poliomyelitis).

Epidemic encephalitis (Encephalitis lethargica).

Yellow fever.

Dengue.

Sandfly fever.

Influenza (Doubtful).

Measles.

Mumps.

Trachoma.

Those producing disease in animals

Pleuropneumonia of cattle.

Foot-and-mouth disease (sometimes transmitted to man).

Canine distemper.

Hog cholera (Swine fever).

Cattle plague (Rinderpest).

Sheep-pox and cowpox.

Psittacosis or parrot fever (Transmissible also to man).

General characters of filtrable viruses:—

Size:—Although they are ultramicroscopic, they vary in size. Thus, the largest form is the virus of pleuropneumonia of cattle, which measures 0·17 to 0·2 μ, being just below the range of visibility (0·2 μ). It should be noted that the unit of measurement employed for these micro-organisms is millimicron ($\mu\mu$) which is equivalent to 1/1,000 μ.

Cultivation:—The filtrable viruses cannot be cultivated *in vitro* by ordinary bacterial methods except the virus of pleuropneumonia of cattle. Recently attempts have been made to cultivate some of them (vaccinia and herpes viruses) in association with living tissues. It is to be noted that colony formation has not been detected.

Effects of physical and chemical agents:—As a rule, these filtrable viruses show increased resistance to glycerine, ultra-violet light and prolonged cold. Thus, they can be preserved in 50 per cent. glycerol at 4°C. for a long time without any loss of virulence, whereas under such circumstances non-sporing bacteria are killed in a short time. They are, however, destroyed by moist heat at 55° to 60°C. in one hour.

Infectivity:—The virus diseases are highly infective and epidemics of many of them (measles, influenza, smallpox, foot-and-mouth disease, etc.) are very difficult to control even with rigid quarantine.

Immunity:—Sera obtained from patients convalescent from diseases due to filtrable viruses have been found to possess marked viricidal properties. Such convalescent sera are being used therapeutically in certain diseases, such as measles and epidemic poliomyelitis. Thus, a high degree of immunity is established in persons surviving the infection and such immunity may last for a long or short period. Active immunisation for the purpose of prophylaxis has also been employed against some of these virus diseases, such as smallpox, rabies, rinderpest and foot-and-mouth disease. It has also been observed that for the proper immunity response, the use of a living virus in an attenuated form is essential.

Cell inclusions:—In many of these virus diseases, when the lesions are examined histologically, peculiar structures are seen inside the cytoplasm or the nucleus of the cell and they fall under two groups:—

(a) *Elementary bodies*—These are supposed to represent the actual virus and are best exemplified by the Paschen corpuscles found in smallpox and vaccinia, globoid bodies in acute anterior poliomyelitis and encephalitis lethargica.

(b) *Inclusion bodies*—These may again be cytoplasmic, e.g., Negri bodies in rabies, Guarnieri bodies in smallpox; or intranuclear, e.g., in the liver cells in yellow fever and in the epithelial cells in herpes.

These inclusion bodies are acidophilic in nature and they may be detected by Giemsa's and Mann's stains which act as useful diagnostic agents.

As to the real *nature of these inclusion bodies*, opinions differ and the following views are held:—

1. That they represent some stage in the development of the viruses themselves.

2. That they consist of a cellular product which surrounds the infective agent as a mantle or sheath—*chlamydozoa* (from *chlamydos*, mantle; *zoa*, animal).

3. That they are regarded as products of cellular reaction or degeneration induced by the action of the virus.

Classification of virus diseases

(Modified from Joseph Fine)

The diseases produced by filtrable viruses can be classified according to their mode of transmission and affinity for particular tissues.

I. Diseases in which the virus is **transmitted by an intermediate insect host,** a dipterous arthropod:—

Yellow fever.
Dengue.
Sandfly fever.

II. Diseases in which the viruses are **transmitted directly.** These fall under the following groups:—

1. *Dermotropic group*, i.e., affecting the skin or mucous membrane only.

Molluscum contagiosum.
Infective warts.
Trachoma.
Foot-and-mouth disease of cattle.

2. *Neuro-dermotropic group,* i.e., affecting the skin and capable of affecting the nervous system.
   - Variola (smallpox).
   - Vaccinia (cowpox).
   - Varicella (chickenpox).
   - Herpes febrilis and herpes zoster.
3. *Neurotropic group,* i.e., affecting the nervous system only.
   - Rabies or hydrophobia.
   - Epidemic poliomyelitis.
   - Epidemic encephalitis or encephalitis lethargica.
4. *Respiratory group,* i.e., affecting mainly the respiratory tract.
   - Rubeola (measles) and rubella (German measles).
   - Influenza.
   - Mumps.
   - Psittacosis.
   - Canine distemper.

SMALLPOX *(Variola)*

Smallpox is an acute, specific, highly infectious disease, characterised by high fever and an eruption on the skin and mucous membranes. The cutaneous eruption appears usually on the third day of the fever and with it the fever shows some remission. The eruption is deep seated and at first consists of small red macules passing through the stages of papules (first to third day), vesicles (third to fifth day) and pustules (fifth to ninth day) and then the eruption begins to desiccate (ninth to seventeenth day). When the eruption becomes pustular, there is a secondary rise of fever. Occasionally, there may be involvement of the central nervous system. In addition to the lesions of the skin and mucous membranes, internal organs are often affected. *Ledingham* has shown that the virus has a marked affinity for the reticulo-endothelial system of the body, and is also harboured by these cells.

Apart from this classical or virulent form of smallpox, another milder form exists which is called *alastrim,* commonly met with in Egypt, West Indies and Brazil.

The **infection of smallpox** has been supposed to take place through the mucous membrane of the upper respiratory tract and is obtained by a direct or indirect contact with a patient. The infectivity of a case of smallpox is greater when the eruption appears and remains active and persists till the scabs have separated. Objects infected [illegible] the sick person are infective and the virus may be transmitted in the [illegible] scale through the air. Hence, persons attending cases of smallpox a[illegible]wn to infect other persons by carrying the germ in their clothings and [illegible] The average *incubation period* of smallpox may be taken as 12 days.

The **causative agent** is a filter-passing virus. Inclusion bodies are found inside the cytoplasm of the epithelial cells in the cutaneous eruption of smallpox and are named after their discoverer as *Guarnieri bodies.* The infective agent is supposed to be present in these bodies and was at one time thought to be a protozoon (*Cytorrhyctes variolæ*). *Pashchen* observed minute round coccus-like granules in the lymph of vaccinia and variolaous lesions. These were called elementary bodies and were supposed to be the virus themselves. It has been further shown that they can be agglutinated by specific anti-variolous serum. The virus of smallpox has been cultivated *in vitro* in association with living and growing tissues. It is extremely resistant to glycerol (50 per cent.) and prolonged cold but is easily destroyed by potassium permanganate in a dilution of 1 in 10,000.

For the **diagnosis of smallpox,** the following laboratory tests have been elaborated:—

(i) Materials taken from the eruptions of smallpox, when inoculated into a rabbit's cornea, produce a characteristic vesicular eruption in 48 hours, which on histological examination reveals the typical inclusion bodies (Guarnieri bodies) in the epithelial cells of the cornea. This is known as *Paul's test* and is an important distinguishing point between variola (smallpox) and varicella (chickenpox).

(ii) *Intradermal test:*—The material from a smallpox vesicle is inoculated into a rabbit intradermally. After a short incubation period, a characteristic inflammatory reaction occurs which may be avoided by injecting along with it the serum obtained from a rabbit hyperimmunised with the vaccinia virus.

(iii) *Flocculation test:*—When an extract of smallpox crusts is mixed with an antivaccinial rabbit serum and incubated at 37°C., a finely floccular precipitate appears. Such a reaction is diagnostic of smallpox.

The virus of smallpox becomes attenuated after its passage through calves. This fact was taken into account by *Pasteur* in the preparation of smallpox vaccine. Such a vaccine, when inoculated into human beings, produces an active immunity which lasts for a period of 5 to 7 years. Hence, to afford continuous protection, revaccination is necessary every 7 years or earlier. The best period for primary vaccination is in infancy at about the *sixth month,* avoiding summer months. The serum of a patient recovered from the disease possesses marked viricidal properties. It should be noted that one attack of the disease often confers a life long immunity. The story of smallpox vaccination has already been referred to on page 117.

The various **methods of prophylaxis** include isolation of the patient, disinfection (formalin appears to be the best disinfectant) and vaccination. Of these, the last method appears to be the safest one for prophylaxis and during an epidemic, all persons coming in contact with a case of smallpox should be inoculated, including the new born babies and even persons who have not been vaccinated within two years. Quarantine period for contacts is 16 days.

Preparation of vaccine lymph:—Healthy female calves (negative to tuberculin test) of 2 to 4 months old are selected for the purpose and a wide area on the posterior of the abdomen and insides of the thighs is scarified with cowpox virus. Very stringent precautions as to cleanliness should be taken. After an incubation period which is about 5 or 6 days, vesicles form. The lymph from these is scraped with a spoon after thorough washing and removing the crusts. The pulpy mass so obtained contains the living virus and is ground up in 50 per cent. glycerol-saline and stored in the dark in an ice chest for some weeks. This process of glycerinisation serves to kill all extraneous organisms except the smallpox virus. After the glycerinated lymph has passed the bacteriological tests, it is distributed in vials or capillary tubes and issued for use. This vaccine lymph, when kept in the dark and cold, remains efficacious for 3 months.

Post-vaccinial encephalitis:—Attention has been drawn lately to a condition of acute disseminated encephalitis or encephalo-myelitis following occasionally 10 to 14 days after primary vaccination and sometimes ending fatally. The origin of this complication is unknown and there are two chief views regarding this. The one is that the condition is due to the neurotropic action of the vaccinia virus. The other is that it is due to another virus lying dormant and only stimulated into activity by vaccination.

Cowpox (*vaccinia*):—This is closely allied to smallpox, the virus being the same. The disease produced in cows is a local one and affects the udder and teats from which human beings and other cows may be infected by direct inoculation. But in none of them is the disease generalised. It has been further observed that persons contracting cowpox are rendered immune to smallpox. This fact was utilised by Jenner in the prophylaxis of smallpox.

CHICKENPOX *(Varicella)*

This disease is also due to a filtrable virus which is quite different from that of smallpox. It is characterised by fever and rashes which appear on the first day of the fever. The eruptions are more superficial than in smallpox and come out in crops, hence they are pleomorphic. The disease is infective during the period of the pre-eruptive as well as eruptive stages. The incubation period of the disease is about 14 to 19 days and quarantine period for contact is about 3 weeks. It should be noted that there is a close association between the virus of chickenpox and that of herpes zoster, and an attack of one disease confers immunity against an attack of the other.

HERPES FEBRILIS

The disease is characterised by a slight febrile attack followed by a vesicular eruption on the skin and mucous membranes of the nose and mouth. It is produced by a filter-passing virus which is present in the clear fluid of the vesicle. The organism can be cultivated artificially in association with living tissues. Injected into rabbits, it produces a fatal form of encephalitis. The virus has been detected in the human [illegible]. Such individuals act as carriers.

HERPES ZOSTER

The disease is characterised by a vesicular eruption along the distribution of peripheral nerve trunks. The attack is preceded by acute hyperæsthesia and severe pain. The condition is associated with an inflammation of the posterior root ganglia. The causative agent is a filter-passing virus and is supposed to be present in a dormant condition in the nervous system and roused into activity by some poison or injury. Etiologically, herpes zoster is quite distinct from herpes febrilis. On clinical grounds it is believed that the viruses of herpes zoster and varicella are closely allied or identical.

RABIES OR HYDROPHOBIA

Rabies is a disease of all mammals, affecting particularly the dogs, jackals and wolves. It is communicated to man by the bite or lick of a rabid animal. The infective virus which is present in the saliva of the rabid animal comes in contact with a cut or abrasion and then penetrates the skin. *Rabies is thus a disease of wound infection.* From the seat of injury the virus, like tetanus toxin, reaches the cord and brain through the nerve fibres. Hence bites on areas, such as the face and hands which are richly supplied with nerves, are most dangerous.

In human beings, the disease manifests itself usually after an incubation period of 30 to 60 days. The first and definite symptom of rabies in man is the apparent fear of drinking water (absent in rabid dogs) for which the name *hydrophobia* is given to this disease. This is due to the convulsive spasms of the pharyngeal muscles. Later, the spasms become generalised, followed later on by paralysis. Mind, however, remains clear quite up to the end. Once developed, the disease is invariably fatal and death may occur at any time through respiratory or cardiac failure.

The **causative organism** is a filter-passing virus. It is easily destroyed by heat and chemicals but is resistant to glycerol. Attenuation of the virus may be brought about by drying in the air over caustic potash or by applying chemical agents, such as phenol. The virulence of the infecting agent (street virus which is found in the naturally infected dogs) may, however, be enhanced by repeated passage through rabbits. In this way a virus is obtained, which kills a rabbit regularly in 6 to 7 days and its virulence cannot be further increased. Such a virus is known as the fixed virus ("*virus fixe*") and is employed in anti-rabic prophylaxis. It should be noted that the virus thus obtained has lost its pathogenicity for dog and man when injected subcutaneously.

Pasteur's method of anti-rabic prophylaxis consists of daily subcutaneous injections of an emulsion of 2 to 3 mm. dried cord taken from a rabbit dying with fixed virus infection. These cords are dried for a period varying from 3 to 14 days and it has been found that a cord dried for 14 days is completely avirulent and deprived of its pathogenicity. For the first inoculation, the cord dried for 14 days is used and in succeeding injections, cords dried for shorter periods are employed. Until required, the dried cords are kept in sterilised glycerine in which the vitality of the virus is preserved and in this way its potency can be maintained upto 20 days.

Semple has modified Pasteur's technique. He gave up the drying method for attenuation but used carbolic acid instead, which decreases the virulence of the virus and ultimately kills it. The advantage of this method is that the infecting element is dead and therefore the vaccine will keep well and can be used anywhere. The introduction of such a vaccine has greatly facilitated the decentralisation of anti-rabic treatment.

Prophylaxis:—As the disease is primarily one of dogs and jackals and secondarily of man, the best way to exterminate rabies is to control the above by quarantine and enforcing the muzzling law. After a person is bitten by a rabid animal, the part should be thoroughly cauterised with nitric or sulphuric acid as early as possible and anti-rabic prophylaxis instituted at once. The long incubation period of the disease offers a chance of carrying out this specific prophylaxis.

Diagnosis of rabies in dogs:—

1. *From the symptoms:*—When a dog has bitten any person, it should be kept under observation at least for 10 days and if no symptoms develop within this period, there is no necessity for undergoing anti-rabic prophylaxis. The symptoms of rabies in dogs are change of temperament in the early stages, restlessness and a tendency to run long distances. The animal becomes paralysed before death. Because of madness, the term *rabies* is applied to this disease.

2. *From the presence of Negri bodies in the central nervous system:*—For this purpose, the animal is killed and the head or brain is sent to the laboratory for examination. Pieces of tissue are taken from the hippocampus major, cerebellum and cerebral cortex. Smears are made by squeezing a portion of the tissue between two slides and stained with Giemsa's or Leishman's stains or by Mann's method. Tissues may be fixed in formalin for histological examination. It should be noted that Negri bodies are difficult to demonstrate in the decomposed brain. Hence the material is to be sent immediately after the animal is killed and when sent to a distance it should be sent in glycerol or packed in ice.

3. *By animal inoculation:*—Emulsion of the brain tissue is prepared in sterile saline and 0·25 c.c. of the emulsion is inoculated subdurally into a rabbit. The typical symptoms of rabies are developed 14 to 21 days after inoculation. Though it is a certain method, it requires much time and therefore is of no value in deciding whether or not specific therapy for prophylaxis should be carried out. To give a negative diagnosis, the inoculated animal should be watched for 2 months and if possible for 6 months.

Negri bodies (Fig. 272).—These are round or oval bodies, measuring usually about 3 to 10 μ in diameter. They stain pink with Romanowsky's stains. They are found as inclusion bodies inside the cytoplasm of the cells of the central nervous system of rabid anim[illegible] specifically abundant in cells of the hippocampus major and Purkinje[illegible] the cerebellum. They were first described by Negri in 1903 and [illegible] named after their discoverer. Though much controversy has taken pl[illegible] the nature of these bodies, whether they are the specific living parasites themselves or simply degenerated cell products, it has been proved beyond doubt that their occurrence is a specific feature of rabies. In consequence, demonstration of Negri bodies in the brains of suspected animals forms the most rapid and accurate method of diagnosis.

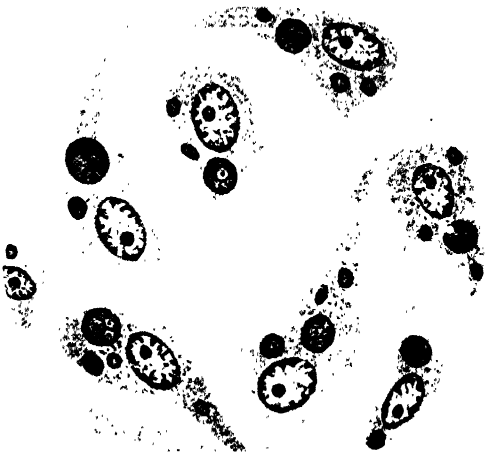

Fig. 272.

Section of hippocampus major from a rabid dog, showing nerve cells containing Negri bodies inside their cytoplasm.

EPIDEMIC POLIOMYELITIS

(Acute anterior poliomyelitis, Infantile paralysis, Heine-Medin disease)

This is a disease chiefly of children. The virus affects the central nervous system with localisation chiefly in the anterior horn cells of the spinal cord. The chief histological change is an acute inflammation with hyperaemia and perivascular cell infiltration. The causative agent is filtrable and is present in the saliva and nasal secretions. Infection is obtained by droplet methods directly from a carrier who harbours the organism in his nose and nasopharynx. The portal of entry is the upper respiratory tract and the virus enters the brain and the cord directly through the olfactory nerve fibres. The disease may be transmitted to higher apes and monkeys. One attack of the disease gives complete immunity for the rest of the life. Serum obtained from convalescent patients possesses viricidal properties and is employed with success in the treatment of the disease during pre-paralytic stage. For this purpose, 30 to 60 c.c. of the serum may be injected intravenously or intrathecally or by both routes.

EPIDEMIC ENCEPHALITIS *(Lethargic encephalitis)*

The disease is due to a filter-passing virus and is characterised by an acute febrile illness with involvement of the upper part of the nervous system (cerebrum, basal ganglia and the brain stem). The disease is prevalent in the mid-period of life. The infection is obtained by the droplet method from healthy carriers and persons in pre-symptomatic stage. The portal of entry is the upper respiratory tract and the virus gains access into the nervous system through the peripheral nerve fibres.

Once the germ is introduced inside the nervous system, it is *"virus en cage"* and does not come out regularly in the saliva as in rabies or in the skin as in herpes. The disease may be experimentally transmitted to monkeys.

YELLOW FEVER

Yellow fever is a disease of tropical and subtropical countries, being particularly prevalent in America and Africa. At present it is unknown in India and the question has been raised as to the possibility of the disease being introduced into this country owing to the rapid transport by aerial route. The causative agent is supposed to be a filter-passing virus. The disease is characterised by high fever, great prostration, bradycardia, tendency to hæmorrhages and jaundice. It is a very fatal disease, death occurring on about the fifth or sixth day of the illness. Infection is conveyed by a blood sucking intermediary, *Aedes ægypti* (Stegomyia fasciata). In order that the insect may become infective, it must feed on the patient during the first 3 days of the illness and after a period of 12 days, it is capable of transmitting the disease to another victim. Excepting the monkey, *Macacus rhesus*, no other laboratory animals can be infected with the virus. Both in man and monkeys, acidophilic inclusion bodies have been detected in the nuclei of the liver cells. The organism has not yet been cultivated. Patients recovering from the disease obtain a life long immunity and the serum of patients convalescent from the disease possesses marked viricidal properties and therefore may be employed for passive immunisation. Active immunisation has also been tried with the emulsion of infected liver or the spleen, the virus being killed by phenol or formalin. The best way to control yellow fever is to attack the breeding places of *Aedes ægypti*.

DENGUE FEVER

The disease is characterised by fever, severe joint pains, backache, headache and aching eyes, a measly roseolar rash on the third or fourth day of the disease and leucopenia. After 2 to 3 days, there is some remission of the fever but the temperature again rises, producing a saddle back temperature chart. The causative organism is a filter-passing virus and is transmitted by *Aedes ægypti*. Like yellow fever, the patient is infective to the mosquito during the first three days of the illness and 12 days must elapse before the insect can transmit the disease to another victim. But once infective, the mosquito will remain so for the rest of its life. The prevention of the disease lies in the destruction of the insect vector, *Aedes ægypti*.

SANDFLY FEVER

This is an acute specific disease characterised by fever of 3 days' duration, bradycardia and leucopenia. The disease is not fatal. It is transmitted by *Phlebotomous papatassi* which becomes infective 7 or 8 days after feeding on a patient. Once infected, it remains so for life. One attack generally confers a life long immunity. The best method of prophylaxis is to guard against the sandfly.

MEASLES *(Rubeola)*

Measles is an acute infectious exanthematous disease of children, characterised by fever, catarrh of the upper respiratory tract with a tendency to pulmonary complications (Streptococcal broncho-pneumonia) and a papular eruption on the skin on the fourth day of the fever. The causative agent is a filter-passing virus but it has not yet been actually identified. It is present in the nasal and nasopharyngeal secretions and infection is obtained directly from a patient during the first four days of the disease, i.e., before the rash appears, after which the infectivity declines. The incubation period is about 9 or 10 days. Serum obtained from a patient convalescent from the disease (the most potent serum being obtained on the sixth to the ninth day after defervescence), when injected intramuscularly into susceptible children, produces an effective passive immunity of short duration (one month). The average dose is 5 c.c. for a child. Such sera may also be used therapeutically. It should be noted that when measles follows serum prophylaxis, the eruptions fail to appear at the site of the injection. This is known as the *Debré phenomenon.* Quarantine period for a measles case is about 14 days.

Rubella (German measles)—The disease is quite distinct from measles and is also due to a filtrable virus. It has a long incubation period, usually 17 or 18 days. There is a short catarrhal stage and the eruption appears within 24 hours. Koplik's spots which are found in the mucous membrane of the mouth in cases of measles are not found in rubella. There is a very marked enlargement of the lymphatic glands.

MUMPS *(Epidemic parotitis)*

It is an acute infectious disease, characterised by fever, swelling of the parotid and other salivary glands and sometimes by a complication of orchitis. Infection is obtained directly from a patient. The organism is supposed to be a filter-passing virus and is present in the saliva. It gains entrance into the salivary glands by way of their ducts.

PSITTACOSIS *(Parrot fever)*

This is primarily a disease of parrots and other birds and man is only secondarily infected. In human beings, the disease is characterised by a typhoid-like fever with pneumonic complication, which often ends fatally. Until recently it was supposed to be due to a Gram-negative bacillus (S. psittacosis), belonging to the Salmonella group. It is now proved to be due to a filter-passing virus. Elementary bodies have also been observed in this disease, which are found inside the reticulo-endothelial system. They are agglutinated specifically by anti-psittacosis serum and actually represent the virus.

FOOT-AND-MOUTH DISEASE

It is an acute contagious disease, occurring in nature amongst cloven-footed animals, chief of which are cattle, sheep, pigs and goats. The disease is characterised by the appearance of vesicles in the fe [illegible] membrane of the mouth, hence the name. Th disease is occasionally transmitted to human beings. The virus is filtrable and has been cultivated artificially.

RICKETTSIA BODIES

This name is attached to a group of minute organisms which are not filtrable but closely related to bacteria. They are found in large numbers in the alimentary canal of some blood sucking insects, such as lice, ticks and bugs. Some of them are definitely related to the causation of certain animal and human diseases. The human diseases produced by these organisms are the following:—

Typhus fever caused by *Rickettsia prowazeki,* transmitted by lice.

Trench fever caused by *Rickettsia quintana,* transmitted by lice.

Rocky mountain spotted fever caused by *Rickettsia rickettsi,* transmitted by tick (Dermacentor venustus). The disease is prevalent amongst wild rodents.

Morphology and staining: —Rickettsiae are small organisms, measuring about 0·3 to 0·5 μ, and appear like small cocci. Bacillary forms may also be encountered. They are arranged usually in pairs but may occur singly or in clusters. They are non-motile organisms. With ordinary aniline dyes, they stain poorly but best results are obtained with Giemsa's stain when they appear purple. They are all Gram-negative.

None of the pathogenic species have been cultivated *in vitro* apart from the living cells.

TYPHUS FEVER

Rickettsia prowazeki (*Rocha-Lima,* 1916) is the causative organism of typhus fever. It is named in honour of the persons who were victims of typhus fever and died while investigating the disease. The latter is characterised by a sudden onset of high fever with severe prostration and an extensive petechial eruption which appears on the fifth day of the disease. The temperature is of a continuous type and terminates by crisis on the fourteenth day. Typhus fever is transmitted by the louse, *Pediculus humanus* (corporis and capitis). In the alimentary canal as well as inside the epithelial cells of the insect, rickettsia-like bodies are seen. The lice become infective from 9 to 12 days after feeding on a patient and remain so for the rest of their lives and even pass on to the next generation. The infection takes place by the contamination of the lice's excreta during scratching. Thus the disease appears to be closely associated with a filthy condition. It may be communicated to anthropoid apes, monkeys and guinea-pigs. The latter animal may be infected by injecting intraperitoneally 2 to 5 c.c. of blood from a patient taken on the seventh to the twelfth day of the disease.

Serum of patients suffering from typhus fever develops the power of agglutinating Proteus X 19 (*vide* page [illegible]) and this agglutination reaction which is employed as a diagnostic me[illegible] known as *Weil-Felix reaction.*

APPENDIX

NORMAL BACTERIAL FLORA OF THE HUMAN BODY *

Alimentary tract:—

Mouth:—

1. Staphylococci.
2. Streptococci of the α-hæmolytic type.
3. Lactobacilli.
4. Spore-bearing aerobes.
5. Organisms of the coliform group.
6. Proteus group.
7. Spirochætes (Treponema microdentium, Treponema macrodentium).

It is to be noted that septic infections in the mouth are relatively rare and are due to the following reasons:—

(a) Flushing action of salivary secretions.

(b) Remarkably good blood supply of the mucosa of the mouth.

(c) Suction currents which draw bacteria directly backwards into the œsophagus.

Gastro-intestinal tract:—The bacterial flora varies with food and age.

At birth—Sterile; infection occurs through food.

Infants when breast-fed:—

1. Bacillus bifidus (99% in early weeks, *Cruickshank*).
2. Enterococci.
3. Organisms of the colon group.

Infants when bottle-fed:—

1. Lactobacillus acidophilus.
2. Enterococci.
3. Organisms of the colon group and spore-bearing group. *Bacillus (Bacteroides) bifidus is uncommon.*

ADULTS:—

Empty stomach—Sterile; because of normal acidity.

Duodenum and upper jejunum—Very few organisms, which consist chiefly of Enterococci.

Lower part of the jejunum and ileum—Numerous organisms are found, such as

1. Enterococci.
2. Staphylococci.
3. Sarcinæ.
4. Yeasts.
5. Lactobacilli.

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*Abridged from Topley and Wilson's "*The principle of bacteriology and immunity*" *Vol. II.*

Large intestine:—

1. Organisms of the colon group (Escherichia coli, Aerobacter ærogenes, etc.).
2. Enterococci.
3. Staphylococci.
4. Clostridium welchii, Clostridium tetani (spores).
5. Lactobacillus acidophilus.
6. Spirochætes.
7. Yeasts.

Less frequent organisms are:—

8. Proteus bacilli.
9. Pseudomonas æruginosa.
10. Klebsiella pneumoniæ.
11. Bacillus subtilis, Bacillus mesentericus.

Respiratory tract:—

Vestibule and anterior part of the nasal fossæ:—

1. Staphylococci.
2. Diphtheroid bacilli.

The remaining part of the nasal fossæ is practically free from organisms and is due to the following reasons:—

(a) Anatomical structure of the nose.
(b) Mechanical action of the mucosa and ciliated epithelium.
(c) Presence of a lytic agent, lysozyme (p. 111), in nasal secretion.
(d) Auto-sterilising power of the nasal mucous membrane.

Accessory nasal air sinuses are normally sterile.

Nasopharynx:—

1. Gram-negative cocci (N. sicca, N. catarrhalis, etc.).
2. Non-hæmolytic streptococci.
3. Diphtheroids.
4. Hæmophilus influenzæ.
5. Diplococcus pneumoniæ (Type IV).

Oropharynx—This part is common to the alimentary and respiratory tracts. Its bacterial flora is the same as that of the nasopharynx.

Larynx, trachea, bronchi, finer bronchioles and alveoli of the lungs are normally sterile. There is no permanent bacterial colonisation of the tracheal and bronchial mucosa and this is because of the following:—

(a) Tortuous nasal passage acting as an efficient filter.
(b) The organisms being removed by the cilia of the lining epithelium.
(c) The organisms being caught up in the mucous secretion and subsequently expectorated.
(d) The organisms are removed via the lymphatics to the regional lymph nodes.

Female genital tract:—

New born children:—

Vulva—Sterile.
Vagina—Sterile.

Adults:—

Vulva:—

1. Streptococcus viridans.
2. Staphylococcus epidermidis.
3. Diphtheroids.
4. Enterococci.
5. Sarcinæ.
6. Gaffkya tetragena.
7. Organisms of the colon group.
8. Mycobacterium smegmatis.
9. Fusiform bacilli.
10. Spirochætes.

Vagina:—

Upper one-third—Sterile.
Lower two-thirds—

1. Döderlein's bacillus.
2. Escherichia coli.
3. Enterococci.

Uterus and Fallopian tubes—Sterile.

External genitalia of both sexes:—

1. Staphylococci.
2. Diphtheroids.
3. Mycobacterium smegmatis.
4. Spirochætes (Treponema refringens, Treponema gracile).

Urinary bladder—Sterile in health.

Skin:—

1. Staphylococci.
2. Diphtheroid bacilli.

Deeper layers of the skin, sweat glands and hair follicles:

1. Staphylococcus epidermidis.
2. Corynebacterium acnes.

External ear:—

1. Staphylococci.
2. Diphtheroid bacilli.
3. Neisseria flava.

Cunjunctival sac:—

1. Staphylococci.
2. Corynebacterium xerosis.

STAINING OF BACTERIA IN TISSUES

Fixation:—Thin pieces of tissues are put in Zenker's fluid for 12 to 24 hours and then washed thoroughly in running water for 24 hours. They are then preserved in 70 per cent. alcohol until used.

Zenker's fluid:—

Pot. bichromate ...	... 2·5 gms.
Hydrarg. perchlor ...	... 5 to 8 gms.
Water	... 100 c.c.

Before use, add 5 c.c. of glacial acetic acid to each 100 c.c. of the fluid.

Paraffin embedding:—Small pieces (3 to 5 mm. thickness) of previously fixed tissue are taken and allowed to pass through the following:—

1. Rectified spirit—24 hours.
2. Absolute alcohol—24 hours.
3. Absolute alcohol with anhydrous copper sulphate—24 hours.
4. Xylol, cedar wood oil, clove oil or terpineol till clear.
5. Paraffin, one change for 2 hours.
6. Paraffin, second change for 2 to 3 hours.

The tissue is then blocked and sections cut in the usual way. For this method, sections should not be more than 3 to 6 μ thick.

Removal of Zenker and paraffin:—

1. Xylol, one change—10 minutes.
2. Xylol, second change—10 minutes.
3. Absolute alcohol, one change—10 minutes.
4. Absolute alcohol, second change—10 minutes.
5. Rectified spirit—5 minutes.
6. 70 per cent. alcohol—5 minutes.
7. Running water—10 minutes.
8. Iodine solution (Lugol's)—10 minutes.
9. Running water—a few seconds.
10. Rectified spirit—5 minutes.
11. Running water—10 minutes.
12. 0·5% sodium hyposulphite solution—5 minutes.
13. Running water—10 minutes.
14. 0·25% potassium permanganate solution—10 minutes.
15. 5% oxalic acid solution—20 minutes.
16. Running water—10 to 15 minutes.

The section is now ready for staining.

Staining of Gram-positive organisms in tissues

Gram-Weigert method:—

Tissues are fixed in Zenker's fluid [illegible] paraffin sections are employed. Remove Zenker and paraffin as [illegible] before.

Stages:—

1. Stain lightly in alum hæmatoxylin.
2. Wash in running water.
3. Put 4% aqueous solution of eosin and allow to act for 5 minutes to half an hour or dilute carbol-fuchsin (1 in 10) for not more than ½ minute, preferably less.
4. Wash in water.
5. Put aniline or carbol methyl violet and allow to act for 5 to 20 minutes.
6. Wash off with water.
7. Put Lugol's iodine and allow it to act for 1 to 2 minutes.
8. Wash off with water.
9. Blot thoroughly and decolourise with several changes of aniline-xylol. Repeat blotting, if necessary. (Aniline-xylol is prepared by mixing equal parts of aniline oil and xylol).
10. Wash off with xylol.
11. Mount in Canada balsam or xylol-colophonium.

Aniline methyl violet:—

Saturated alcoholic solution of methyl violet	10 c.c.
Aniline water (aniline oil—5 parts, water—95 parts; shake thoroughly and filter the milky fluid)	84 c.c.

Pappenheimer's method of staining Gram-positive and Gram-negative organisms in tissues (modified by the senior writer):—

Tissues should preferably be fixed in Zenker's Fluid.

Stages:—

1. Stain section in Sterling's gentian violet for 5 to 15 minutes.
2. With or without washing, pour Gram's iodine and allow to act for 1 to 2 minutes.
3. Wipe off excess of iodine solution all round the section and then decolourise with aniline-xylol to a pale violet colour.
4. Wash off with absolute alcohol quickly and put the section in water.
5. Counterstain with 0·5% saffranin or 1% acidulated neutral red or dilute carbol-fuchsin solution for 1 minute.
6. Wipe off the stain all round the section. Blot thoroughly. Complete dehydration with absolute alcohol as quickly as possible. If necessary, blotting may be repeated.
7. Clear with xylol and mount in Canada balsam.

Acidulated Neutral red solution:—

Neutral red	1 grm.
1% Acetic acid	2 c.c.
Distilled water	1,000 c.c.

Sterling's gentian violet:—

Gentian violet	5 grms.
Alcohol	10 c.c.
Aniline	2 c.c.
Water	88 c.c.

A very ***simple and useful method*** for staining bacteria in tissues found to be very convenient and satisfactory by the senior writer.

Tissues should be fixed in Zenker's fluid and sections must be thin and uniform (Paraffin sections).

Stock solutions:—

Thionine	1 gram.
2·5% solution of carbolic acid in distilled water ...	100 c.c.

For staining:—

Take stock solution	1 part.
Distilled water	3 parts.

Mix well and filter before use.

Stages:—

1. Stain sections in the dilute stain for 10 to 15 minutes.
2. Wash in water.
3. Carefully differentiate in a bowl of water acidulated with a drop of acetic acid.
4. Wash thoroughly in several changes of water.
5. Blot well and then quickly dehydrate with absolute alcohol, clear in xylol, and mount in Canada balsam.

Staining of acid-fast organisms in tissues

Stages:—

1. Stain sections (paraffin sections) with hot carbol-fuchsin for 15 to 20 minutes. Sections may also be kept immersed in the stain in a cylindrical jar in the paraffin oven overnight.
2. Wash in water.
3. Decolourise in 20% sulphuric acid (5% for *M. lepræ*).
4. Wash well in water.
5. Counterstain with methylene blue, brilliant green or malachite green for half to one minute.
6. Wash in water.
7. Blot thoroughly and dehydrate with absolute alcohol.
8. Clear in xylol.
9. Mount in Canada balsam.

In carrying out this stain, handling must be very gentle otherwise the section is apt to get dislodged from the slide.

Staining of Spirochœtes in tissues

Levaditi's method (Original):—

1. Fix sections (1 mm. thick) in 10% formalin for 24 hours.
2. Wash in water for one hour.
3. Place in 96%-98% alcohol for 24 hours.
4. Put in distilled water until the pieces sink.
5. Transfer into a dark bottle containing 1·5 to 3% silver nitrate solution and keep in the incubator at 37°C. for 3 to 5 days.
6. Wash in distilled water for [illegible] hour.

7. Put in the reducing mixture (Pyrogallic acid—4 gms., formalin —5c.c., water—100 c.c.) for 48 hours at room temperature.
8. Wash well in water.
9. Dehydrate in increasing strengths of alcohol and clear in xylol.
10. Embed in paraffin.
11. Cut thin sections and after removing the paraffin with xylol, mount in Canada balsam.

Dobell's method:—

1. Fix tissues in 4% formaldehyde for 1 to 3 days; it may be kept in this solution indefinitely before being stained.
2. Transfer to 95% alcohol and keep it for 24 hours.
3. Pass through decreasing strengths of alcohol to water.
4. Put in 0·25 to 0·5% silver nitrate solution in distilled water in the dark at 37°C. for 18 to 24 hours.
5. Wash in distilled water till it is free from silver nitrate.
6. Put in 0·5 to 1% solution of hydroquinone in 50% alcohol for 18 to 24 hours at room temperature.
7. Wash in 70% alcohol, dehydrate and embed in paraffin in the usual manner.
8. Cut sections, remove the paraffin with xylol and mount in Canada balsam.

Jahnel's method:—

1. Take formol fixed tissues of 2 to 4 mm. thickness.
2. Wash in water for 1 to 3 days.
3. Place in pure pyridin for 1 to 3 days.
4. Wash in many changes of water from 2 to 3 days till all pyridin disappears.
5. Allow to remain for a few days in 5 to 10% formaldehyde.
6. Wash again thoroughly in water.
7. Put in 1% uranium nitrate (Merck) solution in distilled water for half to one hour in the incubator at 37°C.
8. Wash in distilled water for one day.
9. Allow to remain in 96% alcohol for 3 to 8 days.
10. Wash out in distilled water until the block sinks.
11. Place in freshly prepared 1·5% silver nitrate solution in an amber coloured bottle for 5 to 6 days in the incubator at 37°C.
12. Decant the silver nitrate solution and wash quickly in water.
13. Transfer it to the following solution and allow to remain for 1 to 2 days.

(a) Pyrogallol ... 90 c.c. } Decant 15 c.c. of this solution and replace
Acetone ... 10 c.c. } it with pyridin.

or (b) 4% aqueous solution of pyrogallol ... ... 95 c.c.
Formaldehyde ... ... 5 c.c.

14. Wash out in distilled water; embed in paraffiin in the usual manner. Cut thin sections, remove paraffin with xylol and mount in Canada balsam.

Staining of Negri bodies

1. Fix one piece from the Hippocampus Major in *Sansom's fluid:*—

Absolute alcohol	65 c.c.
Glacial Acetic acid	5 c.c.
Chloroform	30 c.c.

Add Hydrarg. perchlor crystals to saturation. Then pass through alcohol, iodine in alcohol (for Hg.) and xylol; make blocks and section.

Mann's stain:—

Eosin 1% (aqueous)	35 parts.
Methyl blue 1% (aqueous)	45 parts.
Water	100 parts.

Stain for 2 to 12 hours.

Rinse in water and wash in pure alcohol.

Then put in *differentiating fluid* (absolute alcohol, 60 c.c. and 1% NaOH in absolute alcohol, 10 m.) until the section becomes red.

Wash out NaOH thoroughly in pure alcohol. Then leave for 2 or 3 min. in acidulated water (1 or 2 minims of glacial acetic acid in 16 ounces of water).

Then pass through pure alcohol and xylol. Mount in Canada balsam.

(*Note*—Methyl blue must be insoluble in pure alcohol. The tinging must be so performed that the chromatin becomes dark blue and Negri bodies bright red).

2. *Smears* from the Hippocampus Major are fixed with Methyl alcohol for two minutes and then stained in the following solution for two minutes:—

2 c.c. stock methylene blue.
0·3 c.c. stock basic fuchsin.
30 c.c. of distilled water.

Wash in water and dry.

Sections also may be stained with the same stain in the same way. *Stock solution:*—

I. Weigh out methylene blue, 1·5 gm., place in a stoppered bottle of 150 c.c. capacity and add 100 c.c. of 90% alcohol. Allow alcohol to remain in contact with the dye for 2 hours, shaking vigorously every few minutes. Filter.

II. Weigh out basic fuchsin, 3·5 gms. and proceed as before.

Smears and sections are also stained [illegible] Giemsa's stain (1 in 20 dilution) overnight. Differentiating fluid, as in Mann's technique, is sometimes used after staining overnight.

For **Hæmatoxylin-Eosin method,** 1% hæmatoxylin in 5% carbolic lotion is recommended.

BACTERIOLOGY OF WATER

Introduction:—With the increasing emphasis on the preventive aspect of modern medicine and the growing demand for protected water supply, bacteriology of water is gaining importance every day.

All natural waters contain bacteria, the variety and number of which may differ according to localities and other varying conditions.

The gross appearance of water is often misleading. Perfectly clear water from wells may be contaminated from neighbouring cess-pools while turbid water of running streams in thinly populated areas may be quite safe.

Sources of contamination:—

Atmosphere:—This always contains a varying number of micro-organisms in suspension, the germ population being larger in crowded cities than in the open country. These bacteria contaminate the rain water which drops from the clouds. As a rule, however, such a pollution is neither very serious nor of any practical importance, as far as health is concerned. In the air, there are hardly any pathogenic micro-organisms and even these are also rapidly killed when exposed to direct sunlight, particularly in the tropics.

Soil:—This forms one of the most important sources of contamination. There is a wide range of bacteria which are normally present in the soil and which are called soil-bacteria. Besides these, a large variety of other organisms is also present in the soil where animal and vegetable matters are decomposing. Gardens, agricultural districts, manured soil, etc., are very rich in this respect. When rain-water flows through the soil, it washes away these materials and thus becomes very heavily polluted. In this respect, the water which flows over the surface is very much worse than that which passes through deeper strata.

Animal and human excreta:—This is the most serious of all bacterial pollutions as pathogenic micro-organisms may contaminate the water through this agency. The dejecta may be actually voided in the water or may reach the water through surface washings. Examples of such a pollution may be seen in rivers flowing through big cities and mill areas. The water becomes heavily contaminated not only through the drainage washings but also through the sewage outfalls. Ponds, tanks, shallow wells, and other collections of surface water are also polluted by washing contaminated garments and fomites.

The greatest danger to man is to be found in a sample of water which is contaminated with human excreta, i.e., fæces, urine and sputum. There is very little risk from water polluted with animal excreta as very few of the infections of the lower animals can be transmitted to man. Water polluted with organic matter of plant origin and with small amounts of inorganic substances in solution is hardly dangerous to man, as far as health is concerned.

Classification:—The bacterial flora of water may be broadly divided into four classes:—

1. **Natural water bacteria**—These are usually derived from the air and dust as well as from the soil through which the water has passed. These are mostly motile bacilli which are divided into liquefiers and non-liquefiers of gelatin, chromogenic bacteria and also sarcinæ and micro-cocci. It is rare to find sterile water in nature. Usually, river and well waters contain natural water bacteria.

2. **Soil bacteria**:—*B. mycoides, B. subtilis, B. mesentericus,* some of the thread bacteria (e.g. Cladothrix dichotoma), etc. These are found in lakes and surface waters and die out rapidly in fairly clean water.

3. **Intestinal bacteria**:—(1) Colon group of bacilli, (2) *Streptococcus fæcalis* (Enterococci), (3) spore-bearing anaerobes, e.g. *Cl. welchii, Cl. sporogenes.* These are not dangerous by themselves when found in water but their presence indicates excretal pollutiton. In routine procedure, Escherichia coli alone is taken as an evidence of fæcal contamination.

4. **Pathogenic bacteria**—*Eberthella typhi, Salmonella paratyphi, Salmonella schottmülleri, Eberthella dysenteriæ* and *Vibrio comma.* These are also of intestinal origin and special techniques are essential for their demonstration.

Many physical and biological factors are concerned with the bacteriology of water. One of the most important determining factors is the presence or absence of sufficient food-supply which usually consists of decomposing animal and vegetable matters. It is for this reason that the number of organisms in surface waters and those of rivers flowing through industrial cities is always very large. Temperature, exposure to light, seasonal conditions and the concentration of mineral salts and acids in the water are also important conditions which limit the number and multiplication of bacteria in water.

It is a common knowledge that sunlight has a purifying influence on surface waters, e.g. ponds, lakes, etc. This beneficial action of the sunlight is utilised by the public health engineers in India. During the preliminary stages in the preparation of filtered water for big cities, the grossly contaminated water from a river is pumped up into large shallow ponds where not only sedimentation of bacteria and other impurities takes place but also a good deal of self-purification occurs under the effects of a strong tropical sun. The bactericidal effects of light are said to penetrate through water to a depth of three feet. With diminishing temperature in the deeper parts, there is a general tendency to a reduction in the number and variety of bacterial flora in water. Bacteria when present in water in a frozen condition are not killed but their further multiplication and growth is inhibited.

A very interesting fact has been noted in connection with the presence of natural bactericidal agents in some river [illegible] In India, the best example is the remarkable destructive effect of the [illegible] of the Ganges and some of its tributaries on bacteria and particularly [illegible]. *comma* (Vibrio choleræ). The cause of such a specific bactericidal [illegible] been found to lie in the presence of *bacteriophage* in water.

Besides these, the phagocytic action of certain protozoa found in water helps considerably to keep the bacterial content low.

Collection and transmission of a sample of water:—A suitable collecting bottle is one with a capacity of 250 c.c. (8 ounces), fitted with a ground-glass stopper, preferably with an overlapping rim. Such bottles are wrapped in paper with a piece of paper between the stopper and the neck of the bottles and carefully sterilised in the autoclave or hot-air steriliser. In collecting samples, utmost care must be exercised to avoid contamination of the water with extraneous bacteria and fingers should not come in contact with the glass-stopper or the neck of the bottle while filling it. The bottle should be filled up completely. Cork stoppers should never be permitted. Also earthen jugs and metal vessels are quite unsuitable.

To get a sample *from a tap*, its mouth should be cleaned with absolute alcohol and the water should be allowed to run off for about 10 minutes before filling the bottle.

If the water is taken *from a tank*, stream or reservoir, the unopened bottle is held in the water at least 10 inches below the surface and then it is filled up. It should be remembered that any stirring up of the sediment at the bottom will enormously increase the bacterial count.

If the water is to be obtained *from a well*, a weighted sample bottle is used. Various devices have been made. A sample bottle weighted with lead attached to the bottom by means of wires passing round the neck will serve the purpose. Two strings are tied, one to the neck and another to the stopper and the whole sterilised. To take the sample, the bottle is lowered to the required depth, and then the stopper is jerked out by means of the other string.

The examination must commence immediately. But it is usually impossible to carry out even the preliminary steps on the spot. In order, therefore, to prevent the multiplication of the bacteria in water during transit, the bottle containing the sample is packed in saw-dust and ice and despatched to the laboratory with the least possible delay. Every sample should be properly labelled with date, time and other details. The examination should not be delayed more than twelve hours.

Methods:—Bacteriological examination of water may be divided into the following:—

I. *Enumeration of the organisms present per c.c.*
   (a) *developing at 37°C.*
   (b) *developing at 20° to 22°C.*
II. *Determination of the chief species present:—*
   (a) *Detection of bacteria of fæcal origin of which Escherichia coli (fæcal) is taken as the standard "indicator" organism.*
   (b) *Detection of pathogenic bacteria e.g. Eberthella typhi, Salmonella paratyphi, Salmonella schottmulleri and Vibrio comma.*

I. Enumeration of the organisms present per c.c.

(a) Two tubes of standard agar medium are melted and kept at 44°C. in a water bath. The sample bottle is shaken vigorously and to the first tube is added 1 c.c. and to the other 0·1 c.c. of water. After thorough mixing, these are poured into sterile plates properly labelled and when the agar has set, the plates are incubated at 37°C. If the sample is suspected to be heavily contaminated, it may be diluted with sterile distilled water in dilution bottles and 1 c.c. of each higher dilution may be put up with the standard agar in the usual way.

(b) In a similar way, two gelatin plates are usually made, the medium being cooled to 30°C. and incubated at 20°C. Gelatin being unsuitable in the hot weather in India and the importance of this portion of the examination lying in the temperature of incubation rather than in the medium used, standard agar may be used instead and incubated at 20°C.

After 24 to 48 hours, the plates are examined and the number of colonies counted, counts should be made on plates showing more than 25 and less than 250 colonies. As an example, if 70 colonies were counted on the agar plate containing 0·1 c.c. of water at 37°C., the number of the bacteria will be 700 per c.c. at 37°C.

II. Determination of the chief species present

(a) *For detection of E. coli* (fæcal), MacConkey's broth is used. The composition is as follows:—peptone 20 gms., sodium taurocholate 5 gms., lactose 15 to 20 gms., neutral red 1% solution 5 c.c., and water 1000 c.c. For technique, *vide* p. 66 with the exception of steps ii and iii. After preparation, the medium is distributed in test tubes in varying amounts according to the strength required. A Durham's tube is included in each tube. The medium may be prepared in double or treble the above strength. Varying quantities of the sample of water are put in broth tubes as follows:—

1 tube containing 10 c.c. of triple strength broth and 20 c.c. of the sample.
2 tubes containing 10 c.c. of double strength broth and 10 c.c. of the sample.
4 tubes containing 5 c.c. of double strength broth and 5 c.c. of the sample.
5 tubes containing 5 c.c. of single strength broth and 1 c.c. of the sample.
5 tubes containing 5 c.c. of single strength broth and 0·1 c.c. of the sample.
5 tubes containing 5 c.c. of single strength broth and 0·01 c.c. of the sample.

The tubes are incubated at 37°C. and reactions are noted at the end of 24 and 48 hours. Each test tube has an inverted fermentation tube and only those that show acid and gas are counted as giving the reaction. In the first three of the above series, each makes up 20 c.c., so that if there is only one tube in the first seven of these that gives the reaction, it means that *Escherichia coli* is present only in 60 c.c. Out of the latter three series, if 3 out of the 5 tubes give the reaction, we consider that *Escherichia coli* is present in the amount of sample put in each tube.

So far we have presumptive evidence of the presence of fæcal contamination. The next procedure is the separation and identification of the lactose-fermenters, i.e., to distinguish E. coli of fæcal origin from the group which is found as saprophytic, having been always present outside the intestinal canal. The organisms belonging to the typical E. coli of fæcal origin should show the following characters (*vide* p. 465):—

Acid and gas in lactose.
Acid and clotting in litmus milk.
No liquefaction of gelatin.
Positive indol reaction.
Positive Methyl red test.
Negative Voges Proskauer reaction.
Negative Koser's test.

For the purpose of obtaining the representative flora, a tube containing a large quantity of water is taken after about 18 hours' incubation as further delay may alter the numerical relations of the various bacilli owing to increasing acidity of the medium by the fermentation of the lactose.

Having selected the tube for sub-culture of the organisms, one loopful of the bile salt broth is taken out from the tube and put straight into 10 c.c. of sterile water (*tube* 1) and well stirred round. With the same loop, a loopful of this dilution is carried into another 10 c.c. of sterile water (*tube 2*) and so on to the *third*. A loopful from tubes 2 and 3 is put on the centre of a plate containing bile salt neutral red lactose agar (*vide* p. 66) and spread out with a sterile glass rod. The plates are incubated at 37°C. for 24 to 48 hours. The foregoing method of dilution usually produces plates containing about 10 to 20 colonies per plate from *tube 3*, and something between 50 and 200 from *tube 2*. Naked eye appearances of the colonies vary a good deal and no reliance can be put on them.

From a good plate, i.e., with 10 to 12 discrete colonies on it, each separate colony is now put through the various biochemical reactions. If instead of discrete colonies there is crowding, it is advisable to pick off 10 or 12 colonies while they are small and inoculate agar slope with each. Eight separate tubes containing the various media mentioned below are inoculated from each colony. The four sugar media (dulcite, adonite, inulin, saccharose) are made up with sugar-free peptone, bile salt and neutral red, according to MacConkey's formula.

A broth tube or peptone water is inoculated for motility and indol production. Buffered glucose broth is used for Voges-Proskauer's reaction and methyl red test. Koser's citrate medium and uric acid medium are also taken for further confirmatory tests. These tubes after inoculation are put up in one rack and incubated for 48 hours at 37°C.

For each sample of water, the results should be tabulated every day by + or − signs and at the end of the test, the reactions should be carefully studied for interpreting the results.

Streptococci—Pure water rarely contains Streptococci and hence the importance of their detection. Source of streptococci in water is often from sewage contamination and hence their presence is always diagnostic of fæcal pollution. The characters of *Streptococcus fæcalis* are as follows: Acid and clot formation in litmus milk, reduction of neutral red and fermentation of lactose, saccharose, mannite and salicin.

Twenty cubic centimetres of the sample are centrifugalised and the deposit plated on brom-cresol purple agar. Also 1 c.c. of various dilutions of water is added to tubes of glucose broth containing an indicator. The broth becomes acid on account of E. coli fermenting glucose and Streptococci tend to outgrow E. coli. The latter may now be plated out and isolated in pure culture.

Search for Streptococci and other fæcal organisms, e.g. *Cl. welchii,* in water is rarely performed in ordinary routine water bacteriology.

(b) *Detection of pathogenic bacteria.*

(1) *Enteric group:*—Isolation of enteric group of organisms from water supplies is very unsatisfactory. *Wilson and Blair's medium* has recently come into prominence and is very useful as a selective medium.

Samples of water may be put on the selective media, such as those of Wilson and Blair, Conradi-Drigalski, malachite green, etc. after concentration which is carried out as follows:—

(i) Boiling in vacuo --Here boiling occurs at a low temperature without killing the organisms.

(ii) Alum precipitation method—To a litre of water are added 5 c.c. of a 10 per cent. solution of alum. After the flocculent precipitate of aluminium hydrate is formed, the bottle is shaken, the precipitate allowed to settle and then centrifugalised at a high speed. The deposit is used for plating. Other media, e.g., brilliant green telluric acid broth, may also be used (*vide* p. 68).

(2) *For dysentery group* of organisms, the same technique is followed.

(3) *Isolation of Vibrio comma from water:*—Concentrated peptone water (peptone 10 gms., sodium chloride 5 gms., water - 100 c.c., pH 8·0) is sterilised in big Ehrlenmeyer flasks and 900 c.c. of the water are added to each of them.

Interpretation of results:—The objects with which the bacteriological examination of water is undertaken are threefold, viz.:—

(1) Potability of water.
(2) Use in commerce.
(3) Efficiency of various purification processes with a view to render polluted water fit for use.

But the ideal would have been reached if the detection of the pathogenic organisms could have been possible by routine everyday methods. Technical difficulties are, however, so enormous and so feeble is their viability outside the body that we have to fall b[illegible]on the total colony count and detection of *Escherichia coli.*

Total colony count affords valuable informations. But alongside with it must be performed the tests for lactose fermenters. It is obvious that a sample containing a large number of saprophytes (e.g. B. subtilis) is less likely to be harmful than a sample containing even a few colonies of *E. coli of fæcal origin*. By comparing the total number of colonies with the number of lactose-fermenters, the relation between the ordinary water-organisms and fæcal ones may be roughly ascertained. Gelatin plates are superior to agar plates for total colonies at 20°C. as they allow all the organisms including saprophytes to grow and differentiate the liquefiers from the non-liquefiers of gelatin. But in hot climates, they are difficult to work with and agar is used instead. In this connection it should be noted that most of the pathogenic organisms grow best at higher temperature, hence agar plates are suitable.

Source of water should also be considered in judging the total colony count. Higher numbers can be permitted for river, shallow well and other upland surface waters but in deep wells, count should be low (e.g. below 10 per c.c.). In good-quality water, ratio of total count at 37°C. and 20°C. should be about 1 20 to 1 50. If, however, the difference is negligible, the water is considered to be very suspicious.

Many authorities have tried to fix some standards for all waters, but every case should be taken up on its own merit. As a working rule, surface waters may be acceptable if the total colony count at 37°C. be not above 100 per c.c., provided that the coli count is also satisfactory. Ground water should show even less than surface water.

Opinion varies greatly on the passable limits of E. coli. Ground water should show freedom from E. coli in 50 c.c. and very deep well waters should be practically sterile (e.g. artesian wells). In surface waters, E. coli should not be present in 10 c.c. samples. Filtered and chlorinated waters should show absence of E. coli in 100 c.c. samples. Absence of Colon bacilli (fæcal type) proves the harmlessness of water as far as bacteriology is concerned. Its absence is a sign of safety while its presence is a danger signal. It is not unreasonable to assume that organisms of intestinal group, such as *E. typhi*, *E. dysenteriæ*, etc., will be absent from a sample free from E. coli.

Final opinion as to the potability and other uses of water should be given after the chemical analysis and examination of the surroundings, if possible. The same water may vary in results according to the season, rains, flooding, etc.

BACTERIOLOGY OF MILK

Milk constitutes a perfect nutritive fluid and may be considered to be one of the best culture media for all kinds of micro-organisms. It is for this reason that even the perfectly pure sample of milk sooner or later shows an abundant growth of contaminating bacteria, the number of which varies, depending on the temperature and the time which elapses between milking and examination. Thus, the study of this subject forms an important matter not only to control the number and nature of its bacterial flora but also to prevent dissemination of many diseases.

In modern times, all civilised countries of Europe and America have realised the enormous importance of the bacteriology of milk in connection with public health and elaborate arrangements have been made to organise, control, and regularly supervise dairy industries. So far as India is concerned, it is a hopeless state of affairs, there being absolutely no control over the supply of milk. This should, however, be immediately taken up by municipal corporations and other public health departments.

Naturally, when the cow is absolutely free from disease, the milk secreted in its breast is perfectly sterile. But it begins to be contaminated as soon as milking is undertaken. The various *sources of contamination* may be considered under the following heads:—

1. **From the cow:**—The skin of the udder may be healthy but soiled with dirt and excreta. This source of contamination may be minimised by observing scrupulous cleanliness during milching. The cow itself may be suffering from some bacterial diseases and the organisms may be discharged through the milk without any evidences of gross disease in the breast. The udder may also be the seat of diseases, such as, cracks, fissures, ulcers, etc.

2. **During milking:** (From the milkman's hands and fingers).—This is one of the most important and serious sources of contamination. The person may be a carrier of some serious disease, such as typhoid fever, cholera, dysentery, tuberculosis, etc., and he may contaminate the milk through his fingers. The organisms will multiply in the milk with astonishing rapidity and when such milk is taken without boiling or without sufficient sterilisation, a local epidemic breaks out.

The vessel in which the milk is collected may contain dirt or may have been washed with grossly impure water and thus contaminates the milk.

3. **After milking:**—After milching, the milk may not be sufficiently protected against bacterial pollution from the atmosphere or from flies. The contamination from the latter often assumes a menacing proportion in India, particularly during the hot season. Another source of contamination, which is very common in India is from impure water which is added to the milk by illiterate and unscrupulous dairy men to dilute their commodity before distribution to consumers.

It should be noted that milk when freshly drawn and kept at a fairly low temperature remains good for a fairly long time but if the sample is boiled, it tends to become bad very rapidly. This quality of the fresh milk to remain "sweet" for a long time is due to the presence of natural bactericidal substances which inhibit the growth of micro-organisms. When boiled, these elements are destroyed and bacteria are then free to grow and multiply in the milk. This property of the milk, though not understood on a scientific basis, has been known to the lay people and hous[illegible]nds in India and other countries from ancient times.

Bacterial flora of milk

NONPATHOGENIC BACTERIA which may be present in milk obtained from healthy cows are the following:—

(1) **Those which produce acid only** are *Streptococcus lacticus* and *Lactobacilli* (*vide* p. 470). These organisms act on the lactose of the milk, producing mainly lactic acid which produces curdling of the milk.

(2) **Those which produce both acid and gas** mainly belong to the *Escherichia coli* group but anaerobic organisms, such as *Cl. welchii*, and *Cl. sporogenes*, may also be present. They ferment the lactose, producing both acid and gas. The milk is clotted by the acid and there is an abundant gas formation.

(3) **Those belonging to the proteolytic group** are the following:—

(a) Spore-bearing aerobic organisms belonging to *B. subtilis* group.

(b) *Proteus vulgaris*.

(c) *Staphylococci* and other micro-cocci.

They also produce curdling of milk but this is brought about by the action of ferments—rennet and casease—secreted by the organisms.

Besides the above, there may be a large variety of other micro-organisms, some of which may act on the protein contents of the milk and render it alkaline, e.g., Alcaligenes faecalis, whilst others may have little or no action and for which they are often called inert bacteria.

PATHOGENIC BACTERIA which may be found in following:—

(1) **Those which are of bovine origin:**—

(a) *Streptococcus hæmolyticus:*—These may be obtained from a cow when the latter suffers from a streptococcal mastitis. There may also be various degrees of ulceration in the udder which may be secondarily infected with hæmolytic streptococci and the latter may be discharged into the milk. Lastly, hæmolytic streptococci may gain entrance into the milk from the throat of a person who acts as a carrier. Such an infected milk has been found to give rise to an epidemic outbreak of sore-throat and scarlet fever.

(b) *Mycobacterium tuberculosis*:—These are usually obtained from a tuberculous ulcer of the udder (*vide* p. 298) which discharges an enormous number of the organisms into the milk. Apart from this, cows suffering from tuberculosis of the internal organs excrete *M. tuberculosis* through the milk, without having any detectable lesion in the udder when the latter is examined either during life or after death. Finally, contamination may also take place from alvine discharges of diseased cattle, particularly the dung, some of which may contain the bacilli. The organisms are always of the bovine type. Although this is often a serious matter in Scotland and other countries, fortunately such a contamination is comparatively uncommon in India.

(c) *Brucella abortus*:—Cows may occasionally suffer from this infection and excrete large numbers of bacilli into the milk which may thus transmit the disease to the consumer. Similarly, in the Mediterranean areas, goats suffering from *Br. melitensis* infection may excrete the bacilli in their milk and thus spread malta fever.

(d) Occasionally, cattle suffering from foot and mouth disease may have vesicles on the udder which may contaminate the milk with the virus.

(2) **Those which are of human origin**:—These are usually derived from a human carrier who may be employed in milching or handling milk. The organism usually contaminates the latter through unclean hands and fingers or in droplets expelled during coughing, sneezing and speaking. The common organisms of this origin are those producing typhoid and paratyphoid group of fevers, dysentery, cholera, acute bacterial food poisoning, scarlet fever, septic sore-throat and diphtheria. It will be seen from the above that contaminated milk is one of the important sources of origin as well as of the spread of many serious diseases.

Bacteriological standard of milk:—There is absolutely no such standard in India, although this should be enforced. This is due to want of legislation regarding the control of milk supplies. Although there is no universally accepted international standard, the public health authorities of many European countries and United States of America have fixed up certain grades with regard to the purity of milk. In Great Britain, the Ministry of Health have set up the following standard for the control of milk supplies:—

1. *Certified milk*:—This milk is produced under the strictest supervision regarding cleanliness as laid down by the laws of the land. The number of bacteria in such milk must not exceed 30,000 per c.c. and Escherichia coli (B. coli communis) must be absent from 0·1 c.c. It must also be obtained from cows which have been found to be free from any tuberculous infection as proved by the tuberculin test.

2. *Grade A milk:*—This milk must not show more than 200,000 organisms per c.c. and E. coli must be absent from 0·01 c.c. The cows must also be free from tuberculous disease.

3. *Grade A milk (pasteurised)*:—The standard is the same as that for Certified milk.

4. *Ordinary pasteurised milk*:—This must not contain more than 100,000 organisms. There is no definite mention about E. coli.

The bacteriological examination of milk:—This is usually carried out on the same lines as the bacteriological examination of water and sewage. It is undertaken to estimate the total bacterial content of a sample of milk, to determine the degree of faecal pollution and to find out whether any pathogenic organisms are present. Of course, the most important of the latter is *M. tuberculosis*.

Collection of milk:—This is very [illegible]ant. It should always be collected in a sterile glass stoppered pl[illegible] about 8 ozs. capacity. The bottle should be filled up completely, put i[illegible] of ice and despatched to the testing laboratory immediately. Delay [illegible]smission may increase the bacterial flora to such a degree as to pres[illegible] entirely different picture.

The **examination** should be undertaken immediately, if possible; at any rate, not more than 24 hours should elapse between milching and examination. The stopper is now removed and the mouth of the bottle having been flamed over the Bunsen burner, the first 2 to 3 ozs. of milk are discarded. After replacing the stopper, the bottle is thoroughly shaken. Now the actual examination is undertaken.

Total bacterial count:—

A. As the number of organisms is very large, suitable dilutions must be made. Usually, a series of 5 dilutions is made as follows:

(1) 1 c.c. of milk + 9 c.c. of sterile distilled water = 1 in 10 dilution.
(2) 1 c.c. of 1 in 10 dilution + 9 c.c. of sterile distilled water = 1 in 100 dilution.
(3) 1 c.c. of 1 in 100 dilution + 9 c.c. of sterile distilled water = 1 in 1,000 dilution.
(4) 1 c.c. of 1 in 1,000 dilution + 9 c.c. of sterile distilled water = 1 in 10,000 dilution.
(5) 1 c.c. of 1 in 10,000 dilution + 9 c.c. of sterile distilled water = 1 in 100,000 dilution.

Always use a separate pipette for each dilution.

A gentle but thorough and prolonged shaking is absolutely essential.

Medium used:

Peptone—5 grams.
Lemco—3 grams.
Agar fibres—20 grams.
Skimmed milk—10 c.c.
Tap water—1000 c.c.

The medium is prepared in the usual way but clearing with egg-white should be avoided. Adjust the reaction to pH 7.6. Plating is done exactly in the same way as in the case of water analysis, using 1 c.c. of the diluted samples. Always make duplicate plates, *i.e.*, for 5 samples of diluted milk, prepare 10 plates. After incubation for 48 hours, the colonies are counted and the total number of bacteria per c.c. may be calculated.

To estimate the amount of fæcal contamination, varying quantities of the milk are added to tubes containing single strength liquid bile-salt-lactose-neutral red medium of MacConkey and incubated for 2 days. Sufficient neutral red should be added, as much of the colour may be masked by the milk. The following procedure may be followed:—

3 tubes containing 1 c.c. each of 1 in 10,000 dilution.
3 tubes containing 1 c.c. each of 1 in 1000 dilution.
3 tubes containing 1 c.c. each of 1 in 100 dilution.
3 tubes containing 1 c.c. each of 1 in 10 dilution.
1 tube containing 1 c.c. of undiluted milk.

If the degree of contamination is suspected to be very high, higher dilutions and lesser quantities may be employed.

Readings are taken after 24 and 48 hours. The tubes which show both acid and gas contain *Escherichia coli* and the tube which shows the above change with the minimum quantity is also determined.

Examination for Pathogenic organisms

Mycobacterium tuberculosis:—This is usually done by centrifugalising a large volume of milk (100 c.c. to 250 c.c.) in special tubes and inoculating guinea-pigs with the sediments as in the case of other tuberculous materials.

Some of the bacilli may be entangled in the fat which collects on the top. Hence, this may be collected and mixed with the sediments. In order to exclude all other micro-organisms which are also concentrated by the process of centrifugalisation and which may be pathogenic to guinea-pigs, the sediments may be treated with antiformin before inoculation into the animal. Film preparations may also be stained for acid-fast bacilli but usually this is of very little use. In this connection it should be remembered that non-pathogenic acid-fast bacilli may occur in milk (*vide* p. 336) and care should be taken to guard against any fallacy. Two animals should be inoculated. One of them may be killed after 4 to 6 weeks and examined for evidences of a tuberculous infection. If the findings are negative, the other animal may be killed another 4 to 6 weeks later.

It is very important to demonstrate *M. tuberculosis* in the guinea-pig's lesion as similar types of pathological changes may be produced in these animals by *Pasteurella pseudotuberculosis* (B. pseudotuberculosis rodentium) and other micro-organisms (*vide* pp. 324, 514).

Other pathogenic micro-organisms which may occur in milk, e.g., typhoid and paratyphoid group of organisms, dysentery bacilli, organisms of food poisoning, Brucella group and Vibrio comma (V. choleræ) can be demonstrated by suitable methods of cultivation appropriate to the particular organism. The centrifugalised deposit should always be used for this purpose. For a detailed study of this subject, special books should be consulted.

Souring of milk:—This is a very important matter in milk industry, involving a considerable amount of economic loss as sour milk is unfit for use in place of ordinary milk. In India and in many other parts of the world, however, sour milk is consumed in large quantities as an article of diet. Of late, a large amount of therapeutic qualities has been attributed to its use in certain chronic intestinal disorders, e.g., dyspepsia, diarrhœa and other chronic infections of the bowels (*vide* p. 472).

The essential principle of this process consists of the activity of the common bacterial flora of milk on certain constituents of the latter. This souring signifies the earliest evidence of bacterial decomposition of milk. The principal ingredient to be acted upon is the lactose which is fermented by the bacteria with the production of a large amount of lactic acid which acts on the calcium caseinogenate of milk, producing casein. The latter, being insoluble, separates out in the form of a flaky or lumpy precipitate which is commonly known as the curd. The most important organisms to bring about these changes are the streptococcus lacticus, the Escherichia acidi lactici (B. acidi lactici) and the lactobacilli, particularly Lactobacillus bulgaricus (B. bulgaricus) and Lactobacilli acidophilus (B. acidophilus). The liquid which separates out is called the whey, which contains some sugar, lactalbumin, lactoglobulin, mineral salts and bacteria. A distinction should be made between sour milk and putrid milk [illegible]. While the former is due to the decomposition of its carbohydrate [illegible] with an acid reaction and is a harmless process, the latter signifies [illegible] decomposition of its protein components with alkaline reaction and [illegible] due to the formation of ammonia and peptone. Putrid milk is [illegible] to health.

Diseases spread by milk:—

I. Milk becoming infected as a result of **disease of the animal:**

1. *Malta fever* (undulant fever).
2. *Abortus fever.*
3. *Bovine tuberculosis.*
4. *Foot-and-mouth disease.*
5. *Streptococcal infection* (cow becoming a "carrier" of the human streptococci).

II. Milk becoming infected **from human sources** either at the farm or at the dairy or during transport or in the household:—

1. *Enteric fever* (typhoid and paratyphoid fevers).
2. *Cholera asiatica.*
3. *Dysentery.*
4. *Food poisoning* (salmonella infection).
5. *Diphtheria.*
6. *Septic sore throat.*
7. *Scarlet fever.*
8. *Infantile paralysis* (acute anterior poliomyelitis).

Methods of sterilisation of milk:—This is an important matter from the public health point of view, as it is the only way to prevent the spread of communicable diseases through milk consumption. In all parts of the civilised world, various processes are employed for this purpose, but the two most important methods are *boiling* and *pasteurisation*. In India, even from very early days, milk is boiled before being consumed. Although this is not by any means a very ideal method, it has undoubtedly been the only effective way of keeping down the very high degree of contamination which is present in Indian milk.

Although boiling kills the bacterial flora of the milk, the process leads to a profound change in its quality. It takes away the flavour of the fresh milk and adds a cooked taste to it. It brings about some change in the composition of the proteins and the salts of calcium, magnesium and phosphorus. It also destroys anti-scorbutic vitamins. Boiled milk undergoes decomposition more readily than fresh milk.

Pasteurisation:—The essential principle of this process consists in exposing the milk to a temperature of 63°C. for half an hour and rapidly cooling it down. By this way all the vegetative micro-organisms are killed while the quality of the milk remains more or less unaltered. Sterilised milk is liable to be contaminated exactly in the same ways as fresh milk and therefore it should be consumed as soon after sterilisation as possible and every precaution should be taken against bacterial contamination during subsequent manipulations.

Condensed milk, dried milk, cream, butter, cheese and similar milk products, all contain varying numbers of bacteria. They may also be contaminated with pathogenic micro-organisms and thus may cause outbreaks of infectious diseases. In this connection, the injurious effects of ice-creams must be particularly remembered. These are often prepared from grossly polluted milk under unclean conditions by men having very little sanitary consciousness. Dangerous microbial agents may thus have an access into these articles of diet. The low temperature only helps to preserve the vitality of these pathogenic organisms for a longer time. The unrestricted sale of these milk products has been found to give rise to serious outbreaks of infectious diseases in many parts of the world and particularly in the tropics.

WASSERMANN REACTION

To test a sample of patient's blood for Wassermann reaction, the following things are necessary:—

(1) Patient's blood.
(2) Complement.
(3) Antigen.
(4) Amboceptor (Anti-sheep).
(5) Sheep's corpuscles.
(6) A known syphilitic serum (S.S.).
(7) A known non-syphilitic serum (N.S.).
(8) Test tubes, 4" × ⅝" sizes.
(9) Test tube racks (zinc and wooden).
(10) Graduated pipettes (1 c.c., 5 c.c., 10 c.c.).
(11) Water bath.
(12) Incubator.
(13) Normal saline.

1. ***Patient's blood:***—About 5 c.c. of blood are drawn out by means of a sterile syringe and are collected in a sterile phial. The blood is allowed to clot when a clear serum separates out. The serum is centrifuged to make it absolutely free from blood corpuscles. Then it is pipetted off into another sterile phial and is inactivated by heating it for 20 minutes to ½ hour in a water-bath at 56°C.

If the syringe is sterilised with alcohol, care should be taken to wash it with normal sterile saline, otherwise the serum will be red (due to the hæmolysis of R.B.C) and anticomplementary. Working dilution of the serum is 1 in 10, usually 0·3 c.c. of the serum and 2·7 c.c. of normal saline are taken.

2. ***Complement:***—For complement, guinea-pig's serum is usually used. Blood is drawn out by puncturing the heart and allowed to clot. The serum is then separated and diluted with normal saline in the proportion of 1 in 10. This diluted complement should be kept either in the ice-chest or in a frigidaire and should be taken out when working. The diluted complement, when exposed to the room temperature for sometime, specially in the tropics, becomes weakened in strength. Working dose is 0.5 c.c. of the diluted complement. It should be remembered that the strength of the complement varies according to the size and health of the guinea-pig and also according to the season.

3. ***Antigens:***—The commonly used ones are Bordet-Gengou (cholesterinised and non-cholesterinised), alcoholic and Noguchi's (a very sensitive antigen, but is no longer used). The antigens which are used at present are four in number, viz., two cholesterinised Bordet-Gengou, one non-cholesterinised Bordet-Gengou and one [illegible] antigen.

Preparation of antigens:—In the preparation of antigens, usually fresh heart muscles are used, either human or bovine, of which the former is preferable. The heart muscle is freed from fat and is then cut into small pieces.

Bordet-Gengou antigen:—Human heart muscle, cut into very small pieces, is put into a clean bottle and absolute alcohol is added, generally in the proportion of 100 to 125, *i.e.*, to 100 gms. of heart muscle, 125 c.c. of absolute alcohol are added. If the alcohol does not cover the heart muscle completely, a few c.c. more may be added. The whole content is shaken daily and is kept at the room temperature for 7 days. After that, the alcohol is thrown away. These pieces of heart muscle are then dried completely, either in the incubator or in a hot-air steriliser. Care should be taken that these pieces are not charred. To these dried pieces, acetone (pure) is added in the proportion of 1 in 2, *i.e.*, to 100 gms. of dried heart muscles, 200 c.c. of acetone are added. The phial with the content is then placed in the cold incubator for seven days and shaken daily. After that, the acetone is thrown away. The pieces of heart muscle are dried as before, and then powdered. The powder is then weighed and double its quantity of absolute alcohol is added. The phial with the content is kept at the room temperature for 3 days and shaken daily. Then the content is filtered and labelled as non-cholesterinised Bordet-Gengou antigen. To make it cholesterinised, add pure cholesterin to the extent of 0·5%.

Alcoholic antigen:—The pieces of heart muscle are weighed and then placed in a clean sterile mortar. Some sterilised sand is added and these pieces of muscle are ground up well by means of a pestle. Absolute alcohol is then added in the proportion of 1 in 10, i.e., if the weight of the heart muscle be 1 gm., 10 c.c. of alcohol are added. The whole content is then thoroughly mixed and poured into a clean sterile glass-stoppered phial. The phial is kept at the room temperature for seven days and everyday the content is shaken for at least one hour. After the seventh day, the content is filtered into another sterile phial and the filtrate is labelled as alcoholic antigen.

Noguchi's antigen:—To a mashed paste of heart muscle, ten parts of absolute alcohol are added. This is kept at 37°C. for several days. An alcoholic extract of heart-muscle is thus obtained. Filter the extract through filter-paper and collect the filtrate. The filtrate is then kept in a shallow dish and evaporated to dryness under an electric fan. The dried residue is then treated with ether, and the ethereal mixture which is turbid is allowed to stand overnight in a covered dish in a cool place. The clear supernatant fluid is decanted off in a clean beaker, from which the ether evaporates, resulting in a concentrated solution. To this, add 10 volumes of pure acetone and mix thoroughly. Let the acetone-insoluble portions settle down as light brownish precipitate, after which the supernatant fluid is decanted off and rejected. This light-brownish precipitate, which on exposure to air becomes sticky, is the Noguchi's antigen.

Stock solution of Noguchi's antigen consists of 0.3 gm. of the precipitate, 1 c.c. of ether and 9 c.c. of methyl alcohol.

Working dilution is 1 in 10 with normal saline.

Note:—Working dose of all antigens is 0·1 c.c. and the dilution depends on the strength of the antigen.

The strength of the antigen or the extent to which the antigen is to be diluted is determined by making a series of dilutions and working with a known syphilitic serum and a known non-syphilitic serum. A proper dilution will be one which with 0.1 c.c. as dose will not show hæmolysis of corpuscles with known syphilitic serum and at the same time will not retard or inhibit hæmolysis in the non-syphilitic serum.

More than one antigen should always be used. The sensitiveness of the test depends upon the sensitiveness of the antigen. It has been found that with the same serum, different results are obtained with different antigens. If a very strong cholesterinised antigen is used, the tendency will be towards a strongly positive reaction, whereas if a weak non-cholesterinised antigen is used, the reaction will be distinctly weaker. Hence in practice, three antigens are used, viz., one strong cholesterinised, one weak non-cholesterinised and a third of moderate strength. The results are somewhat different with each of these antigens. But by taking the average of these three results, a correct conclusion may be arrived at.

4. ***Amboceptor (anti-sheep):***—This is prepared by injecting fresh sheep's corpuscles, washed with normal saline, into a rabbit. Before injection, these corpuscles are diluted with equal parts of sterile normal saline. Generally the ear veins are selected. Number of injections required varies from 6 to 10 and the injections are given on alternate days. After the fifth or sixth injection, the rabbit's blood is tested to see whether any amboceptor has developed, and if so, to what strength. If it develops to a sufficient strength (say about 1 in 500), as much of the rabbit's blood as possible is collected either by puncturing the heart or by dividing the jugular vein. The collected blood is allowed to clot and kept in the frigidaire overnight to get clear serum out of it. This serum is inactivated at 56°C. for half an hour and is labelled as amboceptor.

Titration:—This is done by making a series of dilutions of the serum, say 1 in 100, 1 in 200, 1 in 300 and so on and noting the maximum dilution which in a dose of 0.15 c.c. completely hæmolyses the sheep's corpuscles in a fixed time (say 10 minutes). For instance, if in 1 in 400 dilution, 0·15 c.c. can hæmolyse the sheep's cells in 10 minutes, then the working dilution of this amboceptor will be 1 in 400, keeping the dose as 0.15 c.c. If an amboceptor of known strength is available, it is better to find out the strength of the new amboceptor by titrating it against the amboceptor of known strength.

The dose is 0·15 c.c.

4. ***Sheep's corpuscle:***—Blood is d [illegible] at of the jugular vein of a sheep. It is defibrinated by means of ste [illegible] lass beads in a sterile flask and centrifuged. The plasma is thrown [illegible] nd normal sterile saline is

added to the blood corpuscles settled below. The whole thing is stirred well by means of a sterlie glass rod and is centrifuged again. The corpuscles settle below and the supernatant saline is thrown away. This is repeated twice. Finally a 5% suspension of the corpuscles is made with sterlie normal saline. *The dose is determined according to the strength of the complement.*

OUTLINE OF THE WASSERMANN REACTION

The test is carried out in two stages.

In the *first stage*, the strength of the complement is found out by determining the amount of sheep's corpuscles it can hæmolyse when mixed with antigen, amboceptor, and complement in a given time. This strength of the complement is to be determined every day as already mentioned because its strength varies in different samples.

In the *second stage*, the test proper is carried out with the patient's serum (X), a known syphilitic (S) and a non-syphilitic serum (N.S.). The latter two are necessary because the results are required to be checked every day by comparing with the previously tested sera as controls. Taking all possible precautions about the accuracy of the test, there may still be some factors somewhere which might have escaped notice and vitiated the previous results, giving a clear indication to reject the test and do it over again.

First stage—Determination of the dose of sheep's corpuscles (Fig. 273):—

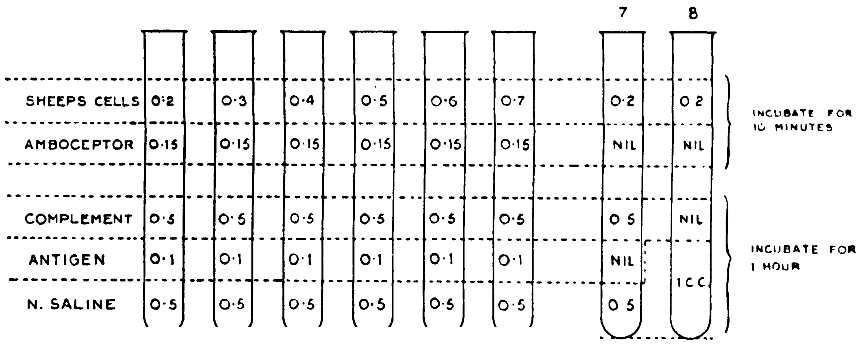

Fig. 273.

Determination of the dose of sheep's cells.

Put up 8 test tubes of $4'' \times \frac{5}{8}''$ sizes in a rack and mark as 1, 2, 3, 4, 5, 6, 7 and 8, keeping 7 and 8 a little apart. Put 0·5 c.c. of normal saline (N. saline) into each of the series of 8 test tubes (this is to make up a convenient volume). Dilute the strongest antigen to its working dilution in a big sized test tube. Put 0.1 c.c. of the antigen into each tube, excepting the last two. Next put 0·5 c.c. of the diluted complement into each of the first seven tubes. The last two tubes, i.e., Nos. 7 and 8, are meant for controls (*vide infra*), the former to represent complement control and the latter to represent control for normal saline that is to be used in the test. Add 0·5 c.c. more of N. saline into tube No. 8. So the volume becomes practically the same in all. Shake all the tubes 1 to 8 thoroughly and put up the whole rack in the incubator at 37°C. for one hour.

After one hour the rack is taken out and 0.15 c.c. of the working dilution of the amboceptor is added to tubes 1 to 6 only. Then put graduated doses of sheep's corpuscles into tubes 1 to 6 as follows:—0.2 c.c., 0.3 c.c., 0.4 c.c., 0·5 c.c., 0·6 c.c. and 0·7 c.c. Into the tubes 7 and 8, put the first dose, namely 0.2 c.c. of the cells. It should be noted that Nos. 7 and 8 will not receive any amboceptor. Then all the tubes are thoroughly shaken and incubated for 10 minutes, after which the rack is taken out and the degree of hæmolysis noted. If the complement is quite strong it may show complete hæmolysis in Nos. 5 or 6 or if it is weaker it will show partial hæmolysis in Nos. 4 and 5 and complete in No. 1 and so on. Then suppose, it shows complete hæmolysis upto tube No. 5, i.e., in the tube in which 0.6 c.c. of the corpuscles has been added, then the working dose of the corpuscles for that day will be 0.5 c.c., i.e., the strength of the red cells just before the tube in which the complete hæmolysis has occurred.

So in this way, the strength of the complement is determined and fixed in terms of hæmolysis of the red blood cells, as the amount of complement has been kept fixed, i.e., 0.5 c.c. of 1 in 10 dilution. The dose of red blood cells varies in different samples of the complement.

Second stage:—Serum to be tested, as already stated, is to be previously inactivated.

In a rack, put 6 big sized test tubes for dilution purposes. Three tubes are meant for three kinds of antigens, say Nos. I, II and III; one tube for known syphilitic serum (S.S.), one for known non-syphilitic serum (N.S.), one for the case (X). Label the tubes according to the materials to be diluted. As for instance, for the dilution of the three antigens, I, II, III and so on. Now dilute with normal saline antigens No. I, II and III in their respective working dilutions. Next dilute S.S., N.S. and serum X 1 in 10 with normal saline (0·3 c.c. of the serum is taken and 2·7 c.c. of normal saline are added to it). In diluting these, the normal saline that is being tested with control in the first stage should be used throughout the process.

Now in another rack, fit up 4 test tubes of the size 4″ × ⅝″ as shown in Fig. 274, one for S.S., one for N.S. and one for X (labelled accordingly) and the fourth one for the hæmolytic system (H.S.) which is to be labelled with

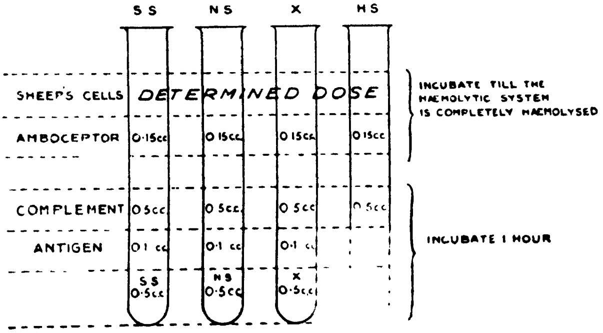

Fig. [illegible]

Scheme of the Wass[illegible]action.

the number of the antigen that will be used in this particular rack. No. I antigen is used in this rack.

Now take two more racks with 4 tubes in each, exactly like the previous one. So altogether three racks are required for three antigens. As already said, each serum is tested with at least three kinds of antigen. So label the fourth tube in the second rack as No. II, because No. II antigen is used in this rack and the fourth tube in the third rack as No. III for the same reason. Now these are the three racks with same sera in all, i.e., the same S.S., N.S. and X but different antigens in each.

Now put 0·5 c.c. of the diluted sera S.S., N.S., and X respectively into tubes allotted for them in all the three sets. Into the tubes which will contain the hæmolytic system, add 0·5 c.c. of N. saline but no serum (this is to make up the volume equal to the serum tubes). Then add 0·1 c.c. of antigen No. I into all the tubes of the first rack, 0·1 c.c. of antigen No. II into all the tubes of the second rack and 0·1 c.c. of antigen No. III into all the tubes of the third rack. Now add 0·5 c.c. of the diluted complement into all the tubes and shake thoroughly.

The tube marked with H.S. in Fig. 274 represents the hæmolytic system. It serves a double purpose. If there is no hæmolysis or partial hæmolysis, it can be concluded that the antigen is responsible for it. It should be noted in this connection, that every antigen destroys the complement to a certain extent (anticomplementary) and when it is too strong its anti-complementary property becomes so marked that it may retard hæmolysis either completely or partially in the hæmolytic system. Hence it will be seen, that this hæmolytic system serves as a control for the antigen.

In this way, when the antigen is found to be very strong and anti-complementary, it should be modified by further dilution. Apart from the above, this hæmolytic system also indicates the time of reading the results of the test (*vide infra*).

Controls:—

It will be evident from the principles of the test, that a positive or a negative result is indicated by the non-hæmolysis or hæmolysis of the sheep's red cells. Apart from the bonafide reaction of the sera, there might be other factors which might be responsible for the production of hæmolysis or non-hæmolysis in the test. If such factors are not eliminated, then it is possible to get a false negative or a false positive reaction. Amongst the factors which will cause a false non-hæmolysis (which is called an anti-complementary reaction), there may be an inherent property of the serum which will destroy the complement in the guinea-pig's serum and consequently indicate a false reaction. Other factors, viz., bacterial contamination, hæmolysed serum, presence of alcohol etc. in the serum, may also destroy the complement, resulting in false positive ractions. To guard against these fallacies, two tubes for each of the serum S.S., N.S. and X are required.

(i) *One tube to show whether the serum is anticomplementary.* Into this tube, we will put the serum, complement, amboceptor and sheep's cells, i.e., a *complete cycle for hæmolysis*. Now, if this tube does not show hæmolysis (which it should have done), it can be concluded that it is the serum which is responsible to bring about this non-hæmolysis; hence the serum is anti-complementary.

(ii) *One tube to show whether the serum is hæmolytic*, i.e., whether it possesses any natural property of hæmolysing the sheep's cells, resulting in a false negative reaction. To check this, put up a tube with serum, complement and sheep's cells, *but no amboceptor*. In a normal serum there should not be any hæmolysis. But if any of these tubes show any hæmolysis, it indicates that the serum contains natural hæmolytic amboceptor and is therefore hæmolytic.

In the case of S.S. and N.S., it is necessary to have these controls each time they are used, because a serum which may not be hæmolytic or anti-complementary at one time may become so on keeping. So for this set, 6 tubes are required—2 tubes for S.S., 2 for N.S. and 2 for X and labelled accordingly. Now for these controls, a special rack, having 2 rows of holes arranged in such a manner that the tubes in the front row do not obstruct the view of those in the back row, is used. The front row having the tubes which will show the anti-complementary controls and the back row will show the hæmolytic controls (Fig. 275). Now put 0·5 c.c. of the respective sera into respective tubes. Then add 0·5 c.c. of the diluted complement into each and shake thoroughly.

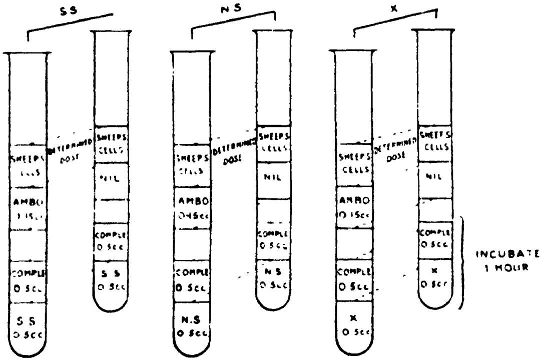

Fig. 275.

Controls for Wassermann reaction.

Next incubate all the 4 racks for 1 hour.

After 1 hour, all these 4 racks are taken out. The dose of sheep's cells that will be required has already been determined in the first stage of the test. Now add 0·15 c.c. of the diluted amboceptor (diluted in its working strength) into each of the tubes of the three racks and only into the tubes of the front row of the control rack. So the control rack will receive amboceptor in the front row only, i.e., the tubes which will indicate the anti-complementary control. Then put the determined dose of the sheep's cells, 0·5 c.c., into all the tubes of all the racks, shake thoroug[illegible] [illegible]t in the incubator and watch constantly for the result. The results w[illegible] [illegible]ecorded when the hæmolytic system in each set will show complete h[illegible]s.

In the rack No. 1, by the time the hæmolytic system tube shows complete hæmolysis, the S.S. should show no hæmolysis, the N.S. complete hæmolysis and the X should show the degree of hæmolysis according to the property of the serum i.e., if the serum is positive it will show no hæmolysis and if it is negative there will be hæmolysis.

The control rack should next be examined.

The front row should show complete hæmolysis, because it contains complement, amboceptor and sheep's cells. If none of the tubes show complete hæmolysis, the serum is said to be anti-complementary, i.e., some factor is present in the serum which is destroying the complement and hence the serum in the racks No. 1, 2 and 3 too will show no hæmolysis and hence will indicate a false positive reaction.

The tubes in the back row ought to show no hæmolysis, because amboceptor has not been added to any of them. If any tube shows hæmolysis, then there is something in that serum which is causing the hæmolysis, though it does not contain a hæmolytic cycle. The corresponding serum in racks No. 1, 2 and 3 will therefore show hæmolysis which it should not do, recording a false result. Thus it is evident, how important these control tubes are as they are the only check against false positive or false negative results.

Reading of the result:—Everything depends upon the degree of hæmolysis. The result of the unknown serum is compared with that of S.S. and N.S. and is expressed according to its degree of hæmolysis on a "plus" sign basis. As three antigens for the same serum have been used, the reaction is recorded with each antigen and the mean result is taken. Suppose the serum X shows the reaction of + + + with antigen No. I, + + with antigen No. II, and + with antigen No. III, then take the mean of these 3 results, i.e., + + or moderately positive.

Method of taking the results:—

+ + +......Strongly positive.
+ + –......Moderately positive.
+ – –......Slightly positive.
– – –......Negative.
±............Should be taken as nega

KAHN'S FLOCCULATION TEST

(For the serum diagnosis of syphilis)

Principle of the test:—This depends on the fact that when the clear serum from a syphilitic patient is mixed with a syphilitic antigen or with the cholesterolised extract of an organ, a white precipitate or visible flocculi occur. Such a flocculation has been found to be specific as it does not take place in the case of a non-syphilitic serum. It is therefore utilised as a substitute for the Wassermann test in the serum diagnosis of syphilis.

Although all the various flocculation tests, such as the Sachs-Georgi reaction, Sigma reaction, Kahn's test, Meinicke's test and Vernes' test, depend on the above ideas with minor individual modifications regarding the preparation of the lipoidal antigen, the Kahn's test is the one which is extensively used both in Europe and America. It has been found, by comparing a very large series of cases with Wassermann's test, that the results are thoroughly dependable and in some cases even better than in the latter.

Advantages:—

(i) The technique and equipments are very simple.

(ii) The test is performed very rapidly.

(iii) In treated cases, the flocculation test may be definitely positive when the Wassermann reaction is weak or negative, i.e., it is more sensitive.

Standard equipments :—

(1) *Antigen suspension tubes*, 5·5 × 1·7 cm. (outside diameter), flat bottomed.

(2) *Test tubes*, 7·5 × 1·2 cm. (outside measurements).

(3) *Test tube racks*—These are made of sheet copper, about 12" long, 3" wide and 2½" high. There are three rows of ten holes, about ½" diameter, in each rack. The row of holes situated in the centre is placed ½" offset from the others.

(4) *Shaking apparatus*—This is adjusted to a speed of 275 to 285 oscillations per minute.

(5) *Standard pipettes*—

(a) for measuring the antigen—
    (i) 1·5 or 1·0 c.c. graduated to 0·05 c.c.
    (ii) 0·25 c.c. graduated to 0·0125 c.c.
(b) For measuring serum—1·5 c.c. graduated to 0·05 c.c. or 1·0 c.c. graduated to 0·01 c.c.
(c) For measuring saline—10 c.c. graduated to 0·1 c.c. or 0·5 c.c.

Other materials :—

ANTIGEN:—This is a specially prepared cholesterolised alcoholic extract of powdered beef heart from which the ether soluble elements have been previously removed.

Note:—As it is rather a very difficult and complicated process to prepare, standardise and titrate the antigen, it is best for a beginner to buy it ready for use. Now-a-days, such prepared antigens are readily available in the market.

SERUM TO BE TESTED:—The serum should be collected exactly in the same way as for the Wassermann test. It should be inactivated at 56°C. for half an hour in a water bath.

Precautions:—Serum should be tested within 48 hours. It should be absolutely clear and free from any turbidity. Although slightly hæmolysed serum may be used, gross hæmolysis is an indication to discard it.

SALINE:—0·9 per cent.

Technique (Standard method)

Get all the equipments ready before preparing the antigen suspension, viz., racks set up, tubes numbered, serum heated and pipettes for measuring the antigen suspension and serum ready. Sometimes, the graduations on the pipettes are not well defined; in that case it is better to mark off the desired measurements with a glass pencil.

Preparation of the standard antigen suspension:—Dilute the stock antigen according to the correct titre which should always be known, by pouring saline on to the antigen as quickly as possible and then back again for five or six times to ensure thorough mixing. For instance, if the titre of the stock antigen is 1 : 1·1, measure 1·1 c.c. of saline into a standard antigen suspension tube and 1 c.c. of stock antigen into another. Pour the saline into the antigen and mix thoroughly as described above. Allow this antigen suspension to stand for ten minutes before use. The suspension should not be used after thirty minutes' standing.

Shake well and distribute in quantities of 0·05, 0·025 and 0·0125 c.c. to each series of three tubes in the metal rack.

Add 0·15 c.c. of the serum to be tested to each of the above antigen tubes and shake vigorously for about ten seconds (by hand) to ensure thorough mixing. Set aside for final three minutes' shaking till the other tests have been done.

Note:—The serum should be added as soon as possible after the antigen suspension has been pipetted.

Controls

Antigen control:—When pipetting the antigen suspension for a series of tests, use the last set of three tubes i.e. 0·05, 0·025 and 0·0125 c.c. as the antigen controls, adding to each tube 0·15 c.c. saline instead of serum. If the antigen suspension is all right, none of these three tubes should show any flocculation.

Serum control:—Each serum should be carefully examined for any particles which might give the appearance of a precipitate. It is essential therefore that the serum used in the test is perfectly clear. In the control, the dilution of the serum with saline may render visible fine particles which were invisible in the undiluted serum. For this purpose, dilute 0·1 c.c. serum with 0·3 c.c. saline, shake the tube for three minutes and examine for particles. If the latter are present, the serum should be cleared by centrifugalisation and retested.

Positive and negative controls:—Always include one or more sera which are known to give positive and negative reactions with each series of tubes for the purpose of checking the results and also for comparison.

Shake the tubes for three minutes in the shaking apparatus. When the latter is not available, shaking may be done by the hand, the speed being approximated as nearly as possible to that of the shaking apparatus.

After the shaking is completed, add 1 c.c. of saline into each tube of the first row, i.e., those containing 0·05 c.c. antigen and 0·5 c.c. to the remaining tubes. Shake sufficiently to mix them well.

Outline of the test

Tube No.	...	... 1	2	3
Antigen	...	... 0·05 c.c.	0·025 c.c.	0·0125 c.c.
Serum	...	... 0·15 c.c.	0·15 c.c.	0·15 c.c.
Shake tubes for three minutes before adding the saline.				
Saline	...	... 1·0 c.c.	0·5 c.c.	0·5 c.c.

Shake well and read the results. In reading the results, the tubes may be held in front of a shaded lamp so that they are illuminated brightly from behind without the glare of the lamp reaching the eyes. If read by direct light, there should not be any strong light and all direct rays of the sun are to be avoided. A shaded northern window is very suitable. The tubes should be held against the light and their opacity noted.

Types of reactions:—The reactions are read on a 'plus' sign basis.

(a) + + + + reaction:—Definitely visible particles suspended in a transparent or opalescent medium. The individual particles are readily visible by direct examination without lifting the tubes from the rack.

(b) + + + reaction:—The particles are definitely visible but less clear cut. They are not always distinguished until the tube is lifted from the rack and examined individually.

(c) + + reaction:—Finer particles suspended in a somewhat turbid medium and distinguished after a careful examination.

(d) + reaction:—Particles are still finer.

(e) ± reaction:—Particles are just within the range of visibility.

(f) – reaction:—No visible particles at all.

In reading the results, Kahn gives the following interpretations—

+ + + +
+ + + } = Positive.
+ +

+
± } = Doubtful.

– = Negative.

Sometimes, in reading the results, it may happen that there is weak or negative reaction in some tube while positive and strong reactions in the others. In such cases, the final result [illegible] be calculated on the average of the number of 'plus' signs represented [illegible] all the three tubes.

DISINFECTANTS

Substances which prevent the growth of bacteria are called *antiseptics* whereas those which actually kill them are called *germicides* or *disinfectants*.

Mode of action of disinfectants:—

(i) By oxidising the protoplasm of the bacteria.

(ii) By coagulating the bacterial protoplasm.

(iii) By combining with the bacterial protoplasm. In this way they act as poisons.

Factors modifying the action of disinfectants:—

(a) Concentration (strength) of the disinfectant—Stronger the solution used, better is the disinfectant action.

(b) Medium in which it acts—Presence of organic matter interferes with the germicidal action to a great extent.

(c) Temperature—The disinfectant action is more effective at higher temperatures.

(d) Time—Longer the time allowed, more effective is the germicidal action.

(e) Type of bacteria on which it acts—Disinfectant action is less effective on spores and spore-bearing bacteria. In this way bacteria vary in their capacity of resisting the germicidal action of disinfectants.

Examples of disinfectants:—

1. Acids and alkalies.
2. Oxidising agents, e.g., hydrogen peroxide, potassium permanganate, etc.
3. Reducing agents, e.g., sulphurous acid.
4. Metallic salts.
5. Halogen and their compounds, e.g., bleaching powder, iodine, chloroform, antiformin, etc.
6. Coal tar derivatives, e.g., phenol, cresol, lysol, izal, cyllin, etc.
7. Aniline dyes, e.g., crystal violet, brilliant green, flavine, etc.
8. Formaldehyde.
9. Alcohol and ether.
10. Essential oils.
11. Soaps.

Note:—Blood when fresh is an excellent germicide.

Standardisation of disinfectants:—There are various methods of testing the power of a disinfectant but the two which are commonly employed are the *Rideal-Walker* and the *Chick-Martin* methods.

THE RIDEAL-WALKER TEST

By this method, the germicidal power of a disinfectant is compared with that of phenol and is expressed as the *carbolic acid co-efficient.* It should be noted that the test requires the finding of the dilution of the unknown disinfectant at which it kills the test organisms in $7\frac{1}{2}$ minutes and this is compared with the phenol dilution under the same standard conditions. The carbolic acid co-efficient is therefore obtained from the following ratio:—

$$\frac{\text{The dilution of the disinfectant which kills } E.\ typhi\ (B.\ typhosus) \text{ in } 7\frac{1}{2} \text{ minutes.}}{\text{The dilution of phenol which kills } E.\ typhi\ (B.\ typhosus) \text{ in the same time.}}$$

Materials required for the test:—

1. Standard carbolic acid solution for control. This is a 5% solution made from pure phenol crystals having a solidifying point of 40°·5C. This should be standardised by titration with decinormal bromine. It keeps indefinitely in stoppered bottles. From this solution, the various working strengths are made up by diluting it with sterile distilled water.

2. Dilutions of the disinfectant X in sterile distilled water.

3. Twenty four hours' broth culture of Eberthella typhi (B. typhosus) —test culture. The organism should be subcultured every day for 7 days and before use it should be shaken and kept aside for half an hour to allow the clumps to settle at the bottom.

4. Standard nutrient broth which is poured in 5 × ⅝ in. test tubes in amounts of 5 c.c. each. The composition of the broth is as follows:—

Liebig's extract of meat	20 gms.
Peptone	20 ,,
Salt (sodium chloride, C.P.)	10 ,,
Distilled water	1,000 c.c..

Apparatus required:—

1. *A special test tube rack*—It contains two tiers, the upper one having holes for 20 test tubes of 5 × ⅝ in. sizes in two rows, each row containing 2 sets of five. This is meant for standard broth tubes (containing 5 c.c. in each) which are numbered 1 to 20 with a grease pencil. The lower tier contains 5 holes for larger sized test tubes which dip into a copper water bath beneath the rack. This is meant for medication tubes, 4 for the disinfectant X and 1 for the phenol.

2. *Inoculating needle*—This consists of a platinum wire of about $1\frac{1}{2}$ inches in length, one end of which is fixed to a glass rod and the other end is made into a loop of 4 mm. internal diameter.

3. *Sterile dropping pipette* for measuring out 0·5 c.c. of broth culture.

4. *Sterile pipettes* and *stoppered measuring cylinders* for obtaining suitable dilutions of the disinfectant to be tested.

5. *Sterile test tubes* (large size).

Technique:—A suitable dilution, say 1 in 100, of standard phenol is made in sterile distilled water. Four dilutions of the disinfectant X to be tested are also made. The dilutions suitable for an accurate test should be determined beforehand by a rough test. Arrange the test tube rack with the tubes as shown in the Fig. 276. The temperature of the water bath is regulated at 15° to 18°C. Five cubic centimetres of the 4 diluted disinfectant and phenol solution are added to the respective large test tubes. At intervals of *half a minute*, 0·5 c.c. of the broth culture of the test organism is added to each of the medication tubes in succession. When the fifth tube has been added to, the first one is exposed to the action of the disinfectant for 2 minutes. After waiting for another half a minute, a loopful of the mixture from the first tube is inoculated into the broth tube No. 1. At intervals of another 30 seconds, a loopful of the mixture from the second medication tube is inoculated into tube No. 2 and so on. In this way the process is repeated at *half minute intervals* till all the broth tubes are inoculated. Thus 4 sets of broth tubes are inoculated at intervals of 2½, 5, 7½ and 10 minutes. All the broth tubes are then incubated at 37°C. for 48 hours and examined for growth. The test is represented graphically as follows:—

5 c.c. of the disinfectant +0·5 c.c. of the test organism.		Time of exposure in minutes and number of broth tubes inoculated.				Incubated at 37°C. for 48 hours.
Disinfectant	*Dilutions*	2½	5	7½	10	
X	*1 in 800*	1 +	6	11	16	,,
X	*1 in 900*	2 +	7 +	12	17	,,
X	*1 in 1000*	3 +	8 +	13 +	18	,,
X	*1 in 1100*	4 +	9 +	14 +	19 +	,,
Phenol	*1 in 100*	5 +	10 +	15	20	,,

Result in the broth tubes is recorded by the '*plus*' and '*minus*' basis. The sign + and – indicating the growth or non-killing and absence of growth or killing of the organisms respectively.

From the above table it will be seen that phenol, 1 in 100 dilution, gives the same result as the disinfectant X in 1 in 900 dilution. The carbolic acid co-efficient of the disinfectant X is therefore $\frac{900}{100} = 9$.

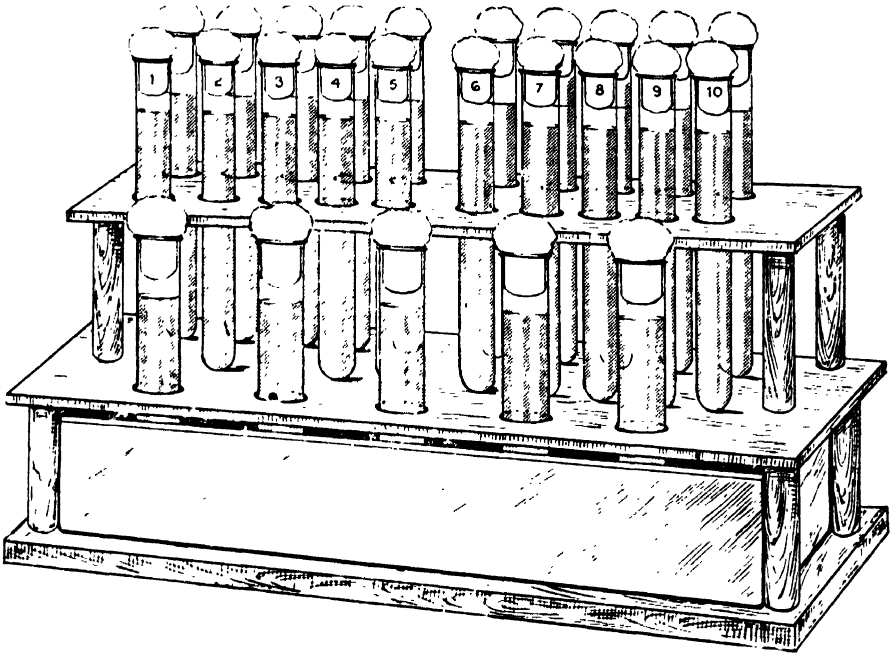

Fig. 276.
Outfit for the Rideal-Walker test.

THE CHICK—MARTIN TEST

In practice, the disinfectant has to act in the presence of organic matter the amount of which considerably modifies the germicidal activity of the disinfectant used. In the Rideal-Walker method the disinfectant acts in an aqueous solution, the only organic matter present being a culture of *Eberthella typhi* (B. typhosus). In the Chick-Martin test, however, the disinfectant is tested on the organism in the presence of a suspension of 3 per cent. dried human fæces at 20°C. and instead of varying the time, it is allowed to act for a constant time (30 minutes), after which subcultures are made into duplicate glucose broth tubes from each dilution.

It should be remembered that the presence of the organic fæcal matter definitely modifies the germicidal action of the disinfectant and the high carbolic acid co-efficients obtained by the Rideal-Walker method are considerably lower when the same material is tested by the Chick-Martin test.

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